

**The Functional Requirement for CD69 in Establishment of Resident
Memory CD8⁺ T Cells Varies with Tissue Location.**

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Abstract

Recent studies have characterized populations of memory CD8⁺ T cells that do not recirculate through the blood but are instead retained in non-lymphoid tissues. Such resident memory CD8⁺ T cells (T_{RM}) are critical for pathogen control at barrier sites. Identifying T_{RM} and defining the basis for their tissue residency is therefore of considerable importance for understanding protective immunity and improved vaccine design. Expression of the molecule CD69 is widely used as a definitive marker for T_{RM}, yet it is unclear whether CD69 is universally required for producing or retaining T_{RM}. Using multiple mouse models of acute immunization, we found that the functional requirement for CD69 was highly variable depending on the tissue examined, playing no detectable role in generation of T_{RM} at some sites (such as the small intestine), while CD69 was critical for establishing resident cells in the kidney. Likewise, forced expression of CD69 (but not expression of a CD69 mutant unable to bind the egress factor S1PR1) promoted CD8⁺ T_{RM} generation in the kidney but not in other tissues. Our findings indicate that the functional relevance of CD69 in generation and maintenance of CD8⁺ T_{RM} varies considerably, chiefly dependent on the specific non-lymphoid tissue studied. Together with previous reports that suggest uncoupling of CD69 expression and tissue-residency, these findings prompt caution in reliance on CD69 expression as a consistent marker of CD8⁺ T_{RM}.

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Chapter 1

Introduction

1.1 Evolutionary Origins of Adaptive Immunity

Vertebrates evolved under constant threat from invading pathogens and with constant exposure to commensal microbes. Prior to the development of modern medical technologies, humans were much more susceptible to death from infectious disease (1). The immune system evolved in this crucible, where exposure to pathogens was often life threatening. Two functionally distinct branches of the immune system operate in concert to help protect organisms from pathogenic threats. The innate immune system defends against infection by recognizing the symptoms of pathogen invasion, such as cellular damage or conserved molecules and structures common among a wide variety of viral or bacterial invaders (2). The adaptive immune system complements innate immunity by providing a mechanism to “remember” previously encountered pathogens, and eliminate them with enhanced and highly specific immunity (3). The specificity and immunological memory of adaptive immunity come with a price, however. The formation of an adaptive immune response is slower to get started, and until it does, the innate immune system must hold the invaders at bay (3). T cells and B cells are the archetypal adaptive immune cells. T and B cells have randomly rearranged receptors (the T cell receptor (TCR) and B cell receptor (BCR)) and the capacity to form immune memory. T cells can be further subdivided into CD4+ and CD8+ subsets, depending on which coreceptor the cells have on their surface.

1.2 The Role of CD8+ T Cells

Cytotoxic CD8+ T cells are one facet of the adaptive immune response. CD8+ T cells recognize peptide antigens being presented in the context of an MHC class I molecule. If an activated CD8+ T cell recognizes the antigen being presented as foreign, it will engage its cytotoxic programming and kill the affected cell. Host cells could acquire foreign peptides for MHC class I presentation upon infection with intracellular microbes, or upon cellular transformation into a

potentially cancer-causing tumor cell. Regardless of the reason, a host cell containing foreign peptides is presumed to be a threat to the integrity of the host organism. Killing the affected host cell is intended to prevent the threat from spreading (4, 5).

Because CD8+ T cells are adaptive immune cells their activity is highly specific. Each CD8+ T cell has a highly specific T cell receptor (TCR), which can interact with peptide MHC class I complexes. During T cell development, their receptors are randomized, with checks to prevent self-reactive T cells from maturing (6).

1.3 The Contribution of CD8+ T cells to Adaptive Immunity

The narrow specificity of adaptive immune cell receptors is foundational to the concept of immunological memory. Prior to exposure to an infectious agent the number of CD8+ T cells that recognize any particular foreign antigen, or precursor frequency, will be low. However, once CD8+ T cells get activated they multiply exponentially generating an overwhelming number of antigen specific cells capable of clearing the infection. After the infection is cleared, these same cells seed a memory population that is much larger than the naïve population was. If the host is exposed to the same pathogen again, these memory cells will be poised to eliminate the threat quickly. This process is immunological memory. It would be impossible for the immune system to predict which pathogens the host will encounter. Instead, the immune system is prepared to recognize and eliminate anything that is not self (6). Once the immune system has been exposed to an invader, it fortifies its defenses against this particular threat.

It would not be feasible for the host to maintain the millions of T lymphocytes expanded over the course of an immune response, nor is it necessary. As an infection or threat is cleared by the immune system the process of contraction greatly reduces the number of antigen specific T cells the host needs to maintain. It falls to the remaining memory T cells to survey the

entirety of the host. Distinct memory subsets keep watch over distinct tissue compartments. Central memory T cells (T_{CM}) reside in the lymphoid tissues and traffic through the blood and lymph. Effector memory T cells (T_{EM}) circulate through some lymphoid tissues (such as the spleen), the blood, and nonlymphoid tissues. Finally, tissue resident memory T cells (T_{RM}) are maintained in the tissue (7). Each of T cell subsets contribute, in their own way, to keep the host protected from reinfection by previously encountered pathogens. As a note, when we subset cells we are sorting cells into distinct boxes as a way of modeling a complex system. Methods like single-cell RNA sequencing have shown us that in practice the lines are a bit blurrier (8). Despite this, cell subsets remain an important tool that helps us model and understand the immune system (8).

1.4 The Tissue Resident Memory Subset

Tissue resident memory CD8⁺ T cells (T_{RM}) are one of the more recently characterized subsets but also one of the most important (9). The defining characteristic of the T_{RM} subset is their pattern of circulation. These cells reside *in situ* and do not recirculate (7). Canonically, T_{RM} were thought to only live in nonlymphoid tissues but recently tissue resident cells have been identified in lymphoid tissues (10). The proximity of T_{RM} to the tissue they protect is central to their role for host defense. Because T_{RM} are located at potential sites of reinfection they are situated to respond immediately. T_{RM} are not dependent on the transit of antigen to far-flung lymph nodes by professional antigen presenting cells. The function of tissue resident cells regarding reinfection is two-fold. Firstly, T_{RM} serve as sentries. Upon reencountering their cognate MHC class I presenting the appropriate peptide antigen, T_{RM} produce inflammatory cytokines like IFN- γ . IFN- γ produced by T_{RM} drives vascular cell adhesion molecule 1 (VCAM-1) mediated recruitment of other immune cells into the inflamed tissue (11). IFN- γ also jumpstarts the immune response, inducing an antiviral state by upregulating interferon-stimulated genes (ISGs) (12). Secondly, like other CD8⁺ immune

memory subsets, T_{RM} are primed to exert their cytotoxic function and eliminate infected cells with precision, using perforin and granzyme B to kill infected cells (9, 13).

The benefits of having T_{RM} in a nonlymphoid tissue are clear, but how do cells become T_{RM} ? At steady state, naïve $CD8^+$ T cells circulate through the blood and secondary lymphoid organs and do not have access to nonlymphoid tissue. To understand how they become activated and gain access to these tissues, eventually becoming tissue resident, it will be helpful to follow the progress of an immune response.

1.5 The Process of Establishing T_{RM}

Imagine a host's nonlymphoid tissue (such as the skin, lungs or a mucosal barrier) has been infected by a virus. Local innate immune cells need to begin producing inflammatory cytokines and chemokines to slow the virus's progress and start the cascade of events that will trigger the adaptive immune response. This inflammatory response will partially be driven by nonimmune parenchymal cells that sense the presence of infection by detecting hallmarks of an invader. For instance the presence of double stranded RNA in mammalian cells indicates the presence of a virus, and the RIG-I pathway has evolved to detect it and subsequently induce an inflammatory response (14). Likewise, widespread cell death and tissue damage signals either the presence of an infection or an opportunity for one to take hold (15). Detecting pattern associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs) alerts the innate immune system to the presence of a threat and induces local innate immune cells such as innate lymphoid cells (ILCs), macrophages, and dendritic cells to begin producing inflammatory cues. Inflammation will help recruit more innate immune cells into the infected site to amplify this innate response even further. Inflammation also induces an antiviral state, making host cells more likely to resist infection.

Dendritic cells and macrophages, or antigen presenting cells (APCs) are of particular interest here as they serve as an interface between the innate and adaptive immune responses. These cell subsets respond to the inflammation and are recruited into the tissue to pick up cell debris and sample potential foreign antigen at the focus of the inflammatory response. These cells proteolyze proteins sampled from phagocytosis, viral infection, or cell transformation to yield short peptides that can be presented on the cell surface in the context of an MHC-I molecule (16, 17). Carrying their peptide cargo, the dendritic cells migrate back to a secondary lymphoid organ, likely the lymph node that drains the infected tissue. There they encounter naïve CD8+ T cells (18).

The adaptive immune response is on the verge of being activated at this point. However, the efficiency and lethality with which it acts would be dangerous for the host if the immune system were to recognize host cells as foreign. To prevent this, in addition to the process of thymic selection, three signals are now required for an antigen presenting cell to activate a CD8+ T cell. The first signal is transmitted via the T cell receptor, which binds to cognate peptide antigen in the context of an MHC molecule on the antigen presenting cell. The second is received through the surface receptor CD28, which interacts with either CD80 or CD86 on the antigen presenting cell. Finally, the third signal is mediated by cytokines such as IL-12 or type I interferons (19, 20). This third signal is partially mediated by the actions of activated CD4+ T cells, which interact with antigen presenting dendritic cells in a CD40 dependent manner (21). The absence of any of these three signals can lead to anergy or cell death instead of a productive immune response.

In order to receive these three signals a naïve CD8+ T cell needs prolonged engagement with the antigen presenting cell. This is where CD69 and its partner S1PR1 play a role in regulating immunity. S1PR1 is a G-protein-coupled-receptor that senses a lipid ligand, S1P (22, 23). S1P is primarily produced by red blood cells (24, 25), the vascular endothelium (26), and the

lymphatic endothelium (27). Immune cells that can sense S1P via expression of the receptor S1PR1 will move towards the chemoattractant. S1P is present in a gradient, with high concentrations in the blood and lymph, and lower concentrations in the parenchyma of lymphoid tissues or nonlymphoid tissues.

If S1PR1 is displayed on the cell surface of a naïve T cell in a lymph node, this cell will detect and be drawn to the higher levels of S1P in the draining lymphatics, causing the naïve T cell to migrate from lymph node to lymph node or back to the circulation (28). However, if there is an active infection, antigen-specific naïve T cells need to stop and form productive interactions with antigen presenting dendritic cells. This is where CD69 steps in. CD69 is a transmembrane protein that directly interacts with S1PR1. In CD8⁺ T cells, CD69 gets upregulated in response to type I interferons or T cell receptor (TCR) signals. In this context, CD69 functions by transiently preventing S1PR1 from reaching the cell surface (29). CD69 upregulation is dependent on both RAS (30) and PKC (31). CD69 is at its highest protein levels 18-24 hours after stimulation, and declines with a half-life of 24 hours (31). The theory is that this gives the cells a window of time to interact with antigen bearing dendritic cells without being drawn out of the lymph node by the S1P gradient (32). If a strong TCR signal is received, this also leads to transcriptional downregulation of S1PR1 (33, 34). S1PR1 downregulation is mediated by PI3K/AKT suppressing FOX1, which was inducing expression of KLF2, which subsequently drove S1PR1 (32). This will hold the cell in place for a prolonged period of time ensuring a productive interaction.

A CD8⁺ T cell that does receive all three of the appropriate signals quickly undergoes a dramatic change in its transcriptional programming to adopt an effector cell fate. Transcription factors including T-bet and Blimp-1 quickly induce the production of effector molecules such as granzyme B and IFN- γ (35). The activated cell begins to proliferate rapidly, with cell divisions occurring every 4-6

hours (4). This transforms a single naïve precursor cell into a massive clonal population of effector cells each capable of recognizing the offending antigen. These cells are primed to act, and no longer require costimulatory signals to be activated (36). This population undergoes metabolic changes as well, dramatically increasing glycolytic and glutaminolytic activity to satisfy the energetic demands of the proliferation and cytotoxic functions (37). Over the course of the next three days or so, the expanded clonal population of cells begins to re-express S1PR1, driving them to leave the lymph node and migrate through the blood (33, 38).

The population of effector CD8⁺ T cells must now find its way into the site of the infection. Upon activation, expression of lymphoid tissue trafficking receptors such as L-selectin and CCR7 are downregulated leading the cells to leave the lymphoid tissue (35, 39, 40). Conversely, expression of chemokine receptors and cell adhesion markers that can draw cells into non-lymphoid tissues are upregulated. These include ligands for E- and P-selectin, $\alpha_4\beta_7$, CCR4, and CCR10 (41). Different surface molecules have different preferences for different nonlymphoid tissue sites (for instance $\alpha_4\beta_7$ canonically draws cells into the intestine (42)) with expression of different markers influenced to a degree by the site of activation (43). Trafficking is generally promiscuous, however, and cytotoxic effector cells are capable of entering a variety of different nonlymphoid tissues regardless of where they first saw antigen (41, 44).

A cytotoxic T cell needs to extravasate, moving from the blood compartment into the nonlymphoid tissue to come into contact with infected cells. A combination of selectins, chemokines and cell adhesion markers mediate this process. Collectively this is referred to as the leukocyte-adhesion cascade (45). Activated T cell selectins are expressed by blood vessels in the nonlymphoid tissues (and upregulated in the presence of inflammation) (46, 47). An activated T cell can bind to these, slowing its progress to a rolling motion in the blood vessel. The chemokines expressed by inflamed tissues and presented by

endothelial cells induce the effector T cell into a crawling motion along the blood vessel. At this point, activated integrins interact with cellular adhesion molecules (including ICAM-I and VCAM-I) to induce arrest, and the effector cell can translocate through the blood vessel endothelium (45). CD69 potentially plays a role in extravasation as well. CD69 binds to myosin 9 and 12, which are both present in the blood vessels of inflamed tissues (48, 49). It is not understood during which phase of the leukocyte-adhesion cascade CD69-myosin interactions occur, but it is likely late in the process as CD69 is not thought to be on the surface of effector CD8+ T cells trafficking through the blood.

After entering the infected tissue effector cells need to come into contact with infected cells in order to exert their cytotoxic function. There is some evidence that inflammatory chemokines help with this process (50), however, their effect is minimal (51) leading to the overall conclusion that contacts with infected cells are stochastic (52). After an effector cell comes into direct contact with an infected cell, an immunological synapse forms. The formation of the synapse is mediated in part by LFA-1-ICAM-1 interactions and partially by interactions between the TCR and peptide-MHC-I complex presented on the surface of the target cell (53). These interactions cause clustering of engaged TCRs and lead to signal transduction events inside the effector CD8+ T cell (54). The ultimate consequence of a successful interaction is the targeted release of perforin and granzyme B which form pores in and subsequently kills the target cell, respectively (55). The Fas ligand pathway is also capable of killing target cells (56), and plays a more or less dominant role depending on the model of infection (57). Finally, IFN- γ and TNF- α are secreted by the activated CD8+ T cell further reinforcing the inflammatory/antiviral cytokine milieu at the site of infection (52). Many effector cells start to upregulate canonical markers of T_{RM} such as $\alpha_e\beta_7$ integrin (α_e is CD103) and CD69 (58). CD103 is upregulated in response to tissue signaling including TGF- β (59) and CCR9 (60). Cells also receive residency signals in the form of IL-33 and type I interferons (61).

Hopefully, at this point the combined efforts of the innate and adaptive wings of the immune system will clear the infection, however, this is not necessary for the formation of tissue resident memory cells.

At the peak of the adaptive immune response more than 10 million antigen specific effector cells could be present (62). It is not sustainable for the host to maintain such a large population of cells after sterilizing immunity is achieved, so the process of contraction begins. Effector CD8⁺ cells express a gradient of the molecule CD127 (IL-7 receptor) and this inversely correlates with KLRG1 expression. Most KLRG1^{hi}CD127^{lo} cells are thought to be more terminally differentiated effectors (Short Lived Effector Cells) that either die off after the immune response or form elements of the T_{EM} compartment. KLRG1^{lo}CD127^{hi} cells (Memory Precursor Effector Cells) are thought to be the precursors of both the T_{CM} and T_{RM} populations (7, 63). Antigen is not required for the eventual formation of T_{RM} (61). In some nonlymphoid tissues (NLT), however, its presence substantially increases the number of T_{RM} (64). T cell tissue residency may be promoted by resident antigen presenting cells. These cells may be a source of antigen and survival signals needed for the cells to transition into long term tissue residency and may help maintain tissue resident memory cells long term (65). As the process of contraction continues tissue signals begin to upregulate a transcriptional program of tissue residency. TGF- β plays a particularly important role, reducing T-bet and downregulating Eomes in nascent T_{RM} (66), continuing to upregulate CD103 (67), and mediating expression of other P and E-selectin ligands (68). Low, but not absent, T-bet expression promotes long term T_{RM} maintenance by driving CD122 (IL-15 receptor β) (66). The transcription factor KLF2 along with its downstream target S1PR1 are repressed by loss of nuclear Foxo1, that is itself induced by cytokine signals transmitted via the PI3K/Akt pathway (69). The end result of this is that the absence of S1PR1 signaling prevents T cells from leaving the nonlymphoid tissue. As time passes the T_{RM} will

begin to adopt a unique transcriptional profile that includes the transcription factors *Hobit* and *Blimp1* (70). *Runx3* is also known to be important (71).

Established T_{RM} are maintained by homeostatic cytokines and signals produced by their tissue niche, but the signals vary with the nonlymphoid tissue. For instance, IL-15 is required for the maintenance of skin T_{RM} (67), but is dispensable for T_{RM} in the intestine (72). It has been proposed that T_{RM} are maintained in sites of tissue repair in the lungs (73) and skin (74, 75) where they interact with antigen presenting cells that maintain an antigen depot (73). It is likely that interactions with other immune subsets contribute to the long-term maintenance of T_{RM} in even more ways that have yet to be elucidated.

1.6 Common Markers of T_{RM}

T_{RM} also express integrins that interact with extracellular matrix proteins in the tissue providing a mechanical means of retention. CD103 is one of the most widely used markers of T_{RM} and is the α_e subunit of the integrin $\alpha_e\beta_7$, which binds to E-cadherin. Expression of CD103 is associated with residency at epithelial

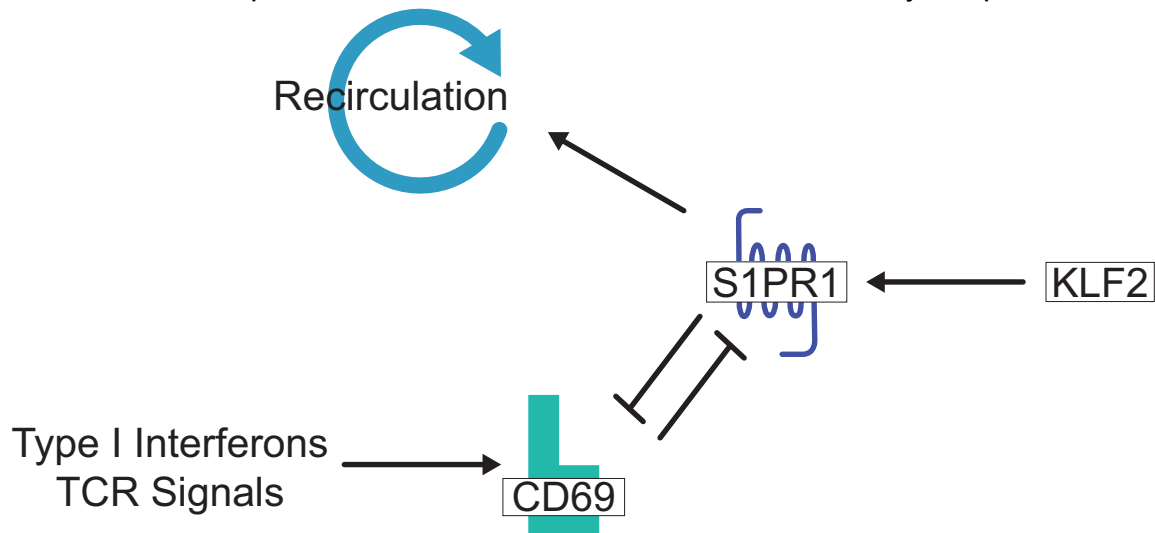


FIGURE 1.1 The hypothesized contribution of CD69 to T_{RM}

The transcription factor KLF2 drives S1PR1 expression, which promotes recirculation. CD69 and S1PR1 are mutual antagonists, with CD69 being induced by TCR signals or type I interferons. Because of its interaction with S1PR1, CD69's presence on T_{RM} was thought to promote tissue residency and CD69 has become one of the most common markers of T_{RM}.

barriers (59). CD49a is another commonly used marker of T_{RM} , and is the $\alpha 1$ component of $\alpha 1\beta 1$, which binds collagen IV (13). Like other antigen experienced T cells, T_{RM} also express the glycoprotein CD44 which interacts with the extracellular matrix and has a role in cell migration (76). Among all of these markers a common thread is direct interactions with extracellular proteins that help to anchor tissue resident cells in place. The canonical role of CD69, the final widely used marker, is distinct.

1.7 CD69 as a Marker of T_{RM}

CD69 begins to appear on the surface of effector cells almost immediately following entry into nonlymphoid tissues. Its surface expression increases over time appearing on most, but not all, T_{RM} at late timepoints (61, 77). CD69 surface expression can be driven by TCR signals (78) and type I interferons (79), but it is not clear if either of these are responsible for CD69 expression on T_{RM} . CD69 directly interacts with and antagonizes S1PR1. It has been hypothesized that CD69's contribution to tissue residency is to prevent S1PR1 from reaching the cell surface, which prevents cells from leaving the tissue (Fig. 1.1) (80), similar to its proposed role during T cell activation in the lymph node as already discussed. CD69 and S1PR1 are mutual antagonists, and KLF2 downregulation is required for the establishment of tissue resident cells (69, 70). This raises the possibility that CD69 expression by tissue resident memory is simply a marker of $KLF2^{low}$, $S1PR1^{low}$ cells, and that CD69's presence is not actually required. It is also possible that CD69 is important for tissue residency in some situations, but not others. Context could be provided by the tissue microenvironment and the source of the antigen stimulus. The focus of this thesis is to determine the functional significance of CD69 expression in a variety of NLT, in the context of varied immune responses.

Chapter 2

A Survey of CD69's Effect on Tissue Residency in Different Contexts

2.1 Introduction

Tissue resident memory CD8⁺ T cells (CD8⁺ T_{RM}) play a key role in protecting non-lymphoid tissues (NLT) from re-infection (7). Expression of the C-type lectin CD69, the integrin chains α E (CD103), and α 1 (CD49a) are often considered definitive markers for typical CD8⁺ T_{RM}. Because CD103 and CD49a are adhesion receptors for the extracellular matrix, their contribution to tissue residency in epithelial tissues is predictable. Yet CD8⁺ T_{RM} in many non-lymphoid sites do not express CD103 and even in NLT where CD103⁺ T_{RM} are abundant, CD103 expression was not always required for their generation (61), suggesting the functional role for CD103 in establishing residency is limited. CD49a is similar. It is not universally expressed, even in epithelial barriers where CD49a⁺ T_{RM} are frequently found (13, 80). CD69 by contrast, is expressed by the vast majority of T_{RM} in diverse NLT, yet its contribution to residency is unclear. Increased cell surface CD69 levels can be driven by either T cell receptor stimulation or certain cytokines (79). CD69 binds and antagonizes the cell-surface expression of G-protein-coupled sphingosine 1-phosphate receptor-1 (S1PR1) in a cell intrinsic manner (29, 79). S1PR1 signaling promotes trafficking towards its lipid ligand, sphingosine 1-phosphate (S1P) which is found in high concentrations in the blood and lymph but much lower concentrations in tissues. In this way, S1PR1 provides a critical mechanism for T cell egress from lymphoid and non-lymphoid sites (81). By inhibiting expression of S1PR1, CD69 can therefore impair egress and promote T cell residency (28, 82). In this way, CD69 expression may promote establishment of resident CD8⁺ T cells in NLT during the acute phase of the immune response. In addition to regulation of S1PR1, other functions of CD69 have been defined, (48, 83) though whether these impact CD8⁺ T cell residency programs are not known. As a result of the widespread expression of CD69 on CD8⁺ T_{RM} and its known effect on S1PR1, many consider CD69⁺ cells (with or without CD103 co-expression) as de facto

tissue resident, and this criteria has been adopted in studies of T_{RM} in mice, humans and non-human primates (84–86).

However, the fidelity of CD69 expression as a critical characteristic of CD8⁺ T_{RM} has been called into question. In the context of LCMV infection, some definitively tissue resident T_{RM} (as defined by parabiosis studies), fail to express CD69 (77). Likewise, several studies in mice and humans showed no increased *Cd69* gene expression in CD8⁺ T_{RM} compared to recirculating memory cells (even, remarkably, when CD69 protein expression itself was used to separate these populations) (70, 85). It is possible, however, that these situations reflect a transient requirement for strong CD69 expression in seeding resident CD8⁺ T cells, and that CD69 expression can subsequently decline in established CD8⁺ T_{RM}. Some studies are consistent with such a model (87). Alternatively, CD69 could be a purely passive marker rather than a functional regulator of tissue-residency. This hypothesis is based on the fact that mutual antagonism of CD69 and S1PR1 for cell-surface expression results in CD69's appearance at the plasma membrane of T cells expressing low levels of S1PR1 (33). The transcription factor KLF2 promotes S1PR1 expression and both S1PR1 and KLF2 are downregulated in CD8⁺ T_{RM} (69, 70, 85) - this loss of expression is functionally important, since sustained expression of KLF2 or S1PR1 blocked establishment of CD8⁺ T_{RM} (69). Hence transcriptional downregulation of S1PR1 could play a key role in establishing residency versus recirculation, with elevated cell surface CD69 expression on T_{RM} simply serving as a marker of S1PR1 low cells, rather than constituting an active player in driving tissue residency.

Still, CD69-mediated inhibition of S1PR1 and transcriptional regulation of S1PR1 might both play critical roles in generation of T_{RM}, perhaps operating sequentially during T_{RM} establishment and maintenance (87). Alternatively, CD69-mediated inhibition of S1PR1 and transcriptional regulation of S1PR1 could be differentially involved in forming T_{RM} depending on the model or specific

NLT studied. As a more definitive test of the mechanistic role played by CD69, various groups have examined T_{RM} generation by CD69-deficient (*Cd69*^{-/-}) CD8⁺ T cells. These studies have yielded mixed results. Some studies indicated that *Cd69*^{-/-} CD8⁺ T cells were severely disadvantaged in generation of CD8⁺ T_{RM} while other reports found a much more moderate effect or a minimal defect in generation of *Cd69*^{-/-} CD8⁺ T_{RM} (67, 73, 87, 88). These studies used a variety of models but focused on a limited set of NLTs, making it difficult to assess the role of CD69 in forming T_{RM} at diverse tissue sites.

To more broadly examine CD69's impact on CD8⁺ T cell residency, we explored generation of T_{RM} by *Cd69*^{-/-} CD8⁺ T cells in multiple NLT, following the response to pathogens that establish tissue-specific or systemic infections, and subunit vaccination.

2.2 Results

2.2.1 CD69 Deficiency Does Not Impact CD8+ T Cell Residency in Many Non-Lymphoid Tissues

Previous studies suggested a role for CD69 in generating site-specific T_{RM} following local infections (the skin after HSV infection and the lung subsequent to influenza infection)(67, 73, 87, 88). Whether there is a requirement for CD69 in establishing T_{RM} in multiple NLT following systemic infections was unclear. Since T_{RM} have been extensively investigated in the context of acute LCMV infection in mice (61, 77), we first used this model. Equal numbers of WT and *Cd69*^{-/-} P14

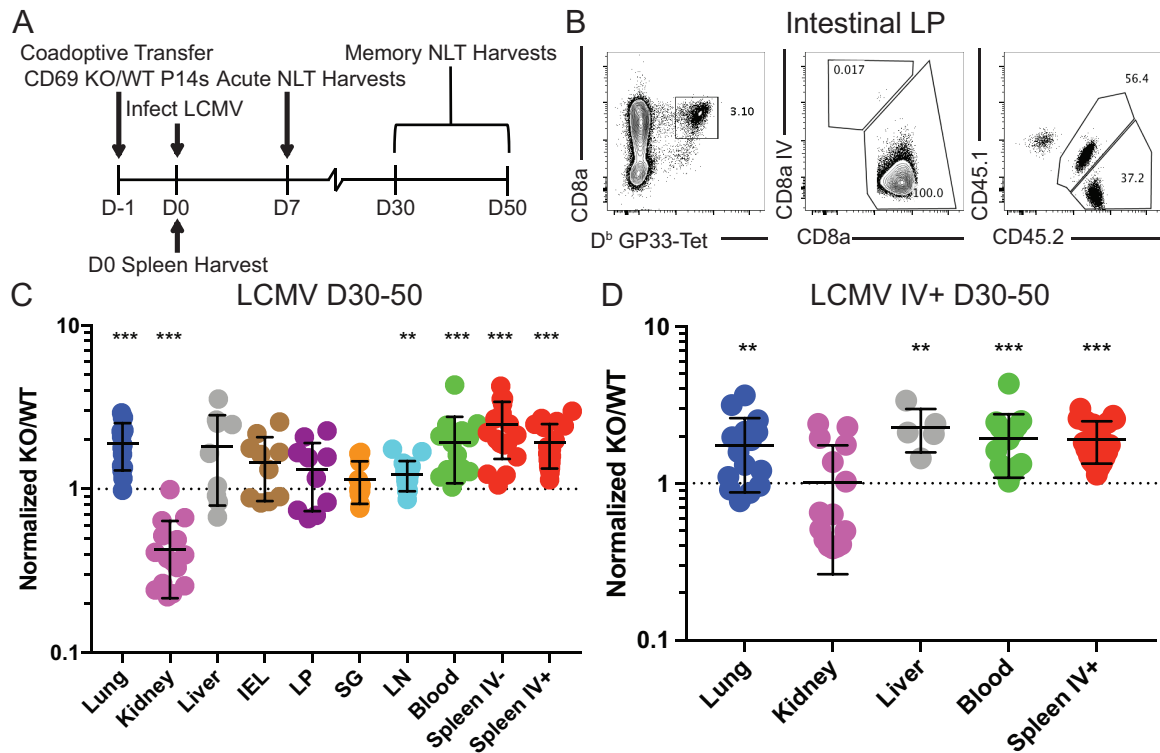


FIGURE 2.1 CD69 plays a minimal role in promoting tissue residency after an LCMV infection

Animals received a co-adoptive transfer of congenically distinct *Cd69*^{-/-} and WT P14 CD8+ T cells. At memory timepoints, lymphocytes were isolated from a variety of different nonlymphoid tissues (A). The gating strategy used to identify D^bGP33-Tet⁺, IV⁻, transferred cells (B). IV labeling was performed with CD8a antibodies that competed with subsequent non-IV CD8a staining. Graphed is the percent of IV⁻ (C) and IV⁺ (D) transferred cells that were *Cd69*^{-/-} over the percent WT, isolated from the indicated tissues days 30-50 p.i. Error bars show mean ± SD, one tailed t test against the known ratio of transferred cells on log₁₀ transformed data.

(LCMV gp33 epitope specific transgenic T cells) CD8⁺ T cells were cotransferred into congenically mismatched recipients, which were subsequently infected with LCMV Armstrong, and the ratio of the CD69-deficient and sufficient cells was determined at various time points in lymphoid and non-lymphoid sites (Fig. 2.1A). Animals underwent intravenous antibody labeling prior to the tissue harvest to distinguish between cells in the vasculature and the tissue parenchyma (89, 90)(Fig. 2.1B). Unexpectedly, analysis at memory timepoints (>day 30) showed minimal differences in the relative proportions of WT and *Cd69*^{-/-} P14 cells in the parenchyma of many tissue sites, although we observed a 3-5-fold disadvantage for *Cd69*^{-/-} P14 in the kidney and a slightly increased proportion of *Cd69*^{-/-} CD8⁺ T cells in the parenchymal cells of the lung as well as among cells within the circulation (blood and spleen; Fig. 2.1C, vascular-associated cells in NLT; Fig. 2.1D). Since the magnitude of these changes were relatively mild, data were aggregated from individual experiments following normalization to the adoptive transfer ratio.

Previous studies suggested that the functional relevance of CD69 may be more substantial during initial establishment of the tissue-resident population (69, 87, 88), therefore we also examined the relative distribution of WT and *Cd69*^{-/-} P14 at the effector phase (day 7) of the LCMV response. Similar to memory-phase, there was minimal impact of CD69-deficiency on representation of P14 cells in the parenchyma of many tissues, although again there was a significant underrepresentation of this population in the kidney (Fig. 2.2A). The magnitude of the kidney residency defect was maintained from this acute timepoint until late memory (Fig. 2.2B). Hence, our data suggest a minimal role for CD69 in generating or retaining tissue-resident CD8⁺ T cells during the response to acute LCMV infection.

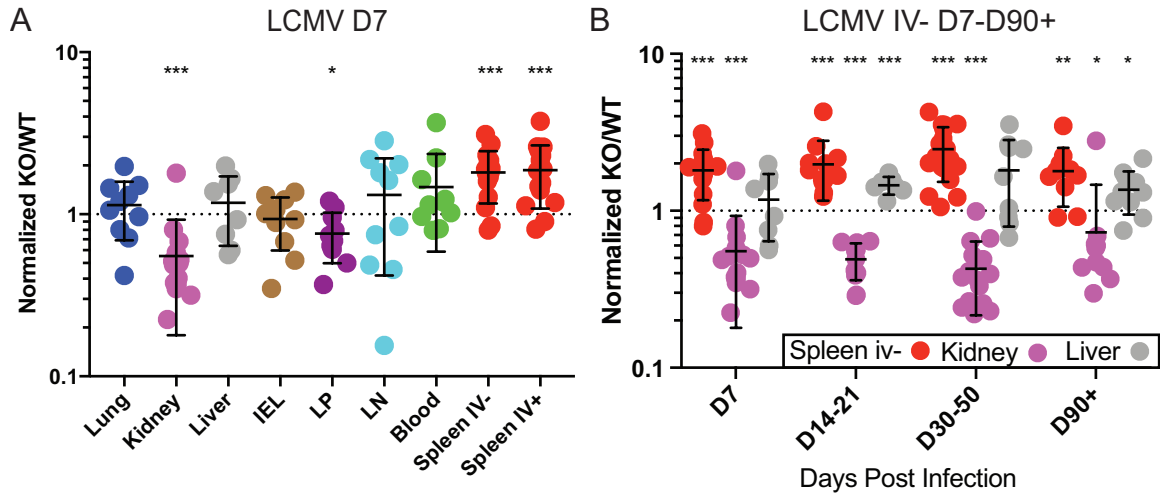


FIGURE 2.2 CD69's effect on tissue residency is maintained over time

Animals received a co-adoptive transfer of congenically distinct *Cd69*^{-/-} and WT P14 CD8⁺ T cells followed by LCMV infection. Graphed is the percent of IV- transferred cells that were *Cd69*^{-/-} over the percent WT, isolated from the indicated tissues days 7 (A) and over a time course p.i. (B). Error bars show mean ± SD, one tailed t test against the known ratio of transferred cells on log₁₀ transformed data.

It was important to investigate whether CD69-deficiency impacted CD8⁺ T cell homeostasis or initial activation, since this could potentially offset a requirement for CD69 in T_{RM} generation. Previous work showed that CD69 deficiency has no discernable effect on CD8⁺ T cell expansion or cytotoxic function following activation (91–93). We observed that CD69 deficiency slightly altered the phenotype but not the numbers of naïve P14 CD8⁺ T cells (Fig. 2.3A, B), that WT and *Cd69*^{-/-} P14 populations showed similar “parking” efficiency following adoptive transfer into animals that were sacrificed prior to infection (Fig. 2.3 C) and that expression of activation and memory markers on WT and *Cd69*^{-/-} P14 CD8⁺ T cells was similar over multiple time points (Fig. 2.3A, D). Finally, it was notable that CD69 expression by WT P14 cells in the blood was essentially undetectable on day 4 of the response to LCMV (when the cells are auditioning for tissue entry (94)), consistent with a negligible impact of CD69-deficiency in these studies (Fig 2.4A).

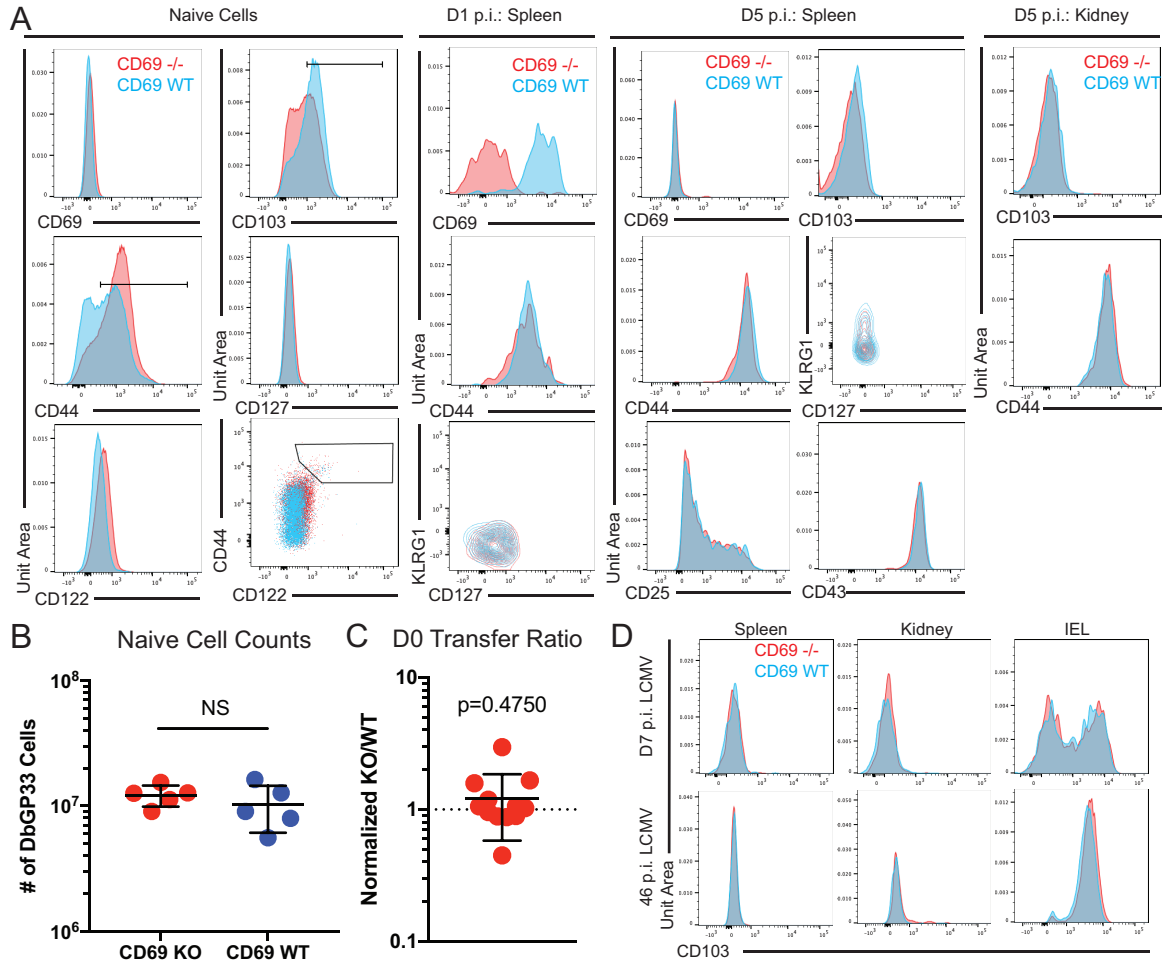


FIGURE 2.3 CD69 does not affect the phenotype of CD8⁺ effectors or T_{RM}
 CD25, CD43, CD44, CD69, CD103, CD122, CD127, KLRG1 on naïve cells, 1, or 5 days p.i. with LCMV in the kidney or spleen, where indicated (A). Number D^bGP33⁺ cells isolated from the spleens of *Cd69*^{-/-} or WT animals (B). The normalized ratio (*Cd69*^{-/-}/*CD69* WT) of cells recovered from the spleens of mice that received a co-adoptive transfer one day prior with no other treatment (C). Surface expression of CD103 in the spleen, kidney, and IEL either 7 or 46 days p.i. with LCMV (D). Error bars show mean \pm SD, two tailed t test for (B), one tailed t test against the known ratio of transferred cells on log₁₀ transformed data for (C).

In addition to acting as cytotoxic effectors, T_{RM} serve as immune sentinels at barrier surfaces (95). To test the alarm functions of T_{RM}, we evaluated peptide rechallenge via the female reproductive tract (FRT) (11). Animals received a cotransfer of WT and *Cd69*^{-/-} P14s, followed by LCMV infection. At memory timepoints, *Cd69*^{-/-} P14s were present at slightly reduced frequency in the female

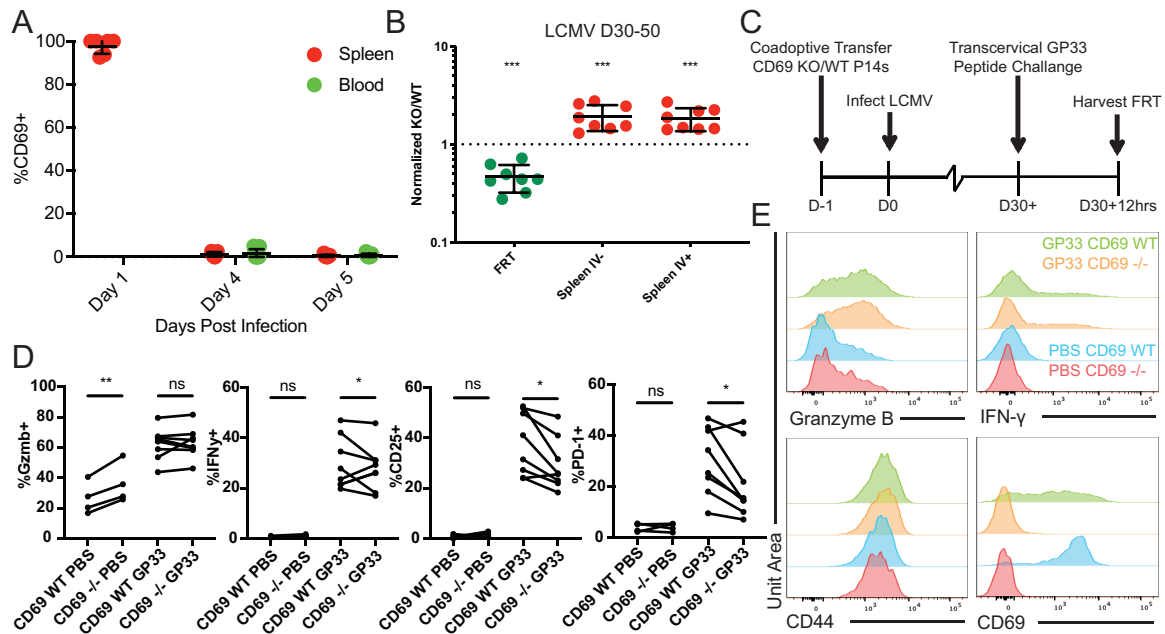


FIGURE 2.4 CD69 does not impact effector functions of T_{RM}

The percent of CD69 WT co-transferred cells expressing CD69 (gates set on *Cd69*^{-/-} cells) at the indicated timepoints, in the indicated tissues post LCMV infection (A). Animals received a co-adoptive transfer of congenically distinct *Cd69*^{-/-} and WT P14 CD8⁺ T cells followed by LCMV infection. At memory timepoints lymphocytes were isolated from the whole female reproductive tract and spleen of mice that did not receive additional treatment (B). A similar cohort of animals received transcervical re-challenge with gp33 peptide or PBS at memory timepoints with isolation of lymphocytes from the FRT 12hrs post treatment (C). Quantified expression of Granzyme B, IFN-γ, CD25, and PD-1 (D). Representative expression of Granzyme B, IFN-γ, and CD44 on *Cd69*^{-/-} or WT cells isolated from the FRT of LCMV immune animals transcervically challenged with either gp33 peptide or PBS control (E). Error bars show mean ± SD, one tailed t test against the known ratio of transferred cells on log₁₀ transformed data for (B), paired two tailed t test for (D).

reproductive tract (FRT), similar to what was observed in the kidney (Fig. 2.4B, 2.2A). To assess the function of the FRT WT and *Cd69*^{-/-} P14 T_{RM}, a cohort of memory-phase animals was transcervically challenged with cognate gp33 peptide (or PBS as a control) to activate FRT T_{RM} (Fig. 2.4C) and quantified Granzyme B, IFN-γ, CD44, CD69, CD25, and PD-1 expression on the P14 population 12 hours later using flow cytometry (Fig. 2.4D, E). *Cd69*^{-/-} P14s exhibited strong induction of activation markers, IFN-γ production and upregulation of the cytolytic molecule Granzyme B, albeit some of these responses were modestly altered compared to WT P14 cells in the same site.

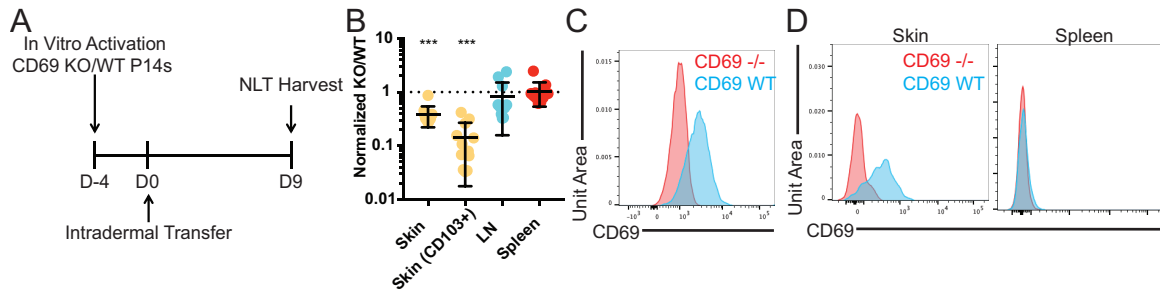


FIGURE 2.5 Absence of CD69 impairs T_{RM} formation following intradermal coadoptive transfer

Congenically distinct *Cd69*^{-/-} and WT CD8⁺ P14 T cells were activated *in vitro* with αCD3/αCD28/IL-2 for 4 days followed by a co-adoptive intradermal transfer into recipient animals (A). The normalized ratio of *Cd69*^{-/-} over WT cells isolated from the indicated tissues 9 days after intradermal co-transfer with the ratio of transferred cells indicated by the dotted line (B). Expression of CD69 on cells at the time of cotransfer (C), and on cells isolated from the skin and the spleen 9 days post transfer (D). Error bars show mean ± SD, one tailed t test against the known ratio of transferred cells on log₁₀ transformed data.

Hence our data indicate that, despite reduced frequency of *Cd69*^{-/-} P14 T_{RM} in the FRT, these cells exhibit a potent recall response.

Previous studies, using a distinct *Cd69*^{-/-} strain, had reported substantial defects in generation of skin *Cd69*^{-/-} T_{RM} following intradermal injection of *in vitro* activated CD8⁺ T cells (87). Indeed, we found that CD69-deficiency led to a substantial reduction in the generation of CD103⁺ skin T_{RM} following this approach (Fig. 2.5A, B). Our findings therefore support earlier conclusions on a role for CD69 in controlling CD8⁺ T cell tissue residency in some assays (87), and hence validate the impact of CD69-deficiency in our studies. It is important to note, however, that in this model CD69 expression by WT cells is clearly detectable at the time of injection (Fig. 2.5C) which contrasts with the low expression of CD69 on blood cells following LCMV infection (Fig. 2.4A). Such data suggest that intra-dermal studies exaggerate the role of CD69, with respect to cell-surface CD69 at the time of NLT entry.

2.2.2 The Site of Infection Dictates CD69's Impact on Tissue Residency

It was possible that the limited role for CD69 in CD8⁺ T cell tissue residency we observed following LCMV infection (including the reduction in kidney *Cd69*^{-/-} T_{RM}), was unique to the tissue-tropism of that pathogen. Previous reports showed that CD69 deficiency impairs tissue residency in the lungs after influenza infection (88), and the skin after HSV infection (67, 87), consistent with a role for CD69 being contingent on the site of tissue tropic infection. Hence, we extended our studies to assess the impact of CD69-deficiency on the response to influenza, using a recombinant PR8 strain expressing the gp33 epitope, recognized by P14 CD8⁺ T cells (Fig. 2.6A). In agreement with previous studies (73, 88) we found that *Cd69*^{-/-} P14 T_{RM} were under-represented in the lung following influenza infection (Fig. 2.6B). This contrasts with our findings after LCMV infection, where *Cd69*^{-/-} cells were overrepresented among the lung parenchymal pool (Fig. 2.1C, 2.2A), although previous studies suggest this population is not truly resident (96). However, similar to our findings with LCMV, influenza infection resulted in reduced accumulation of *Cd69*^{-/-} P14 CD8⁺ T cells in the kidney (Fig. 2.6B).

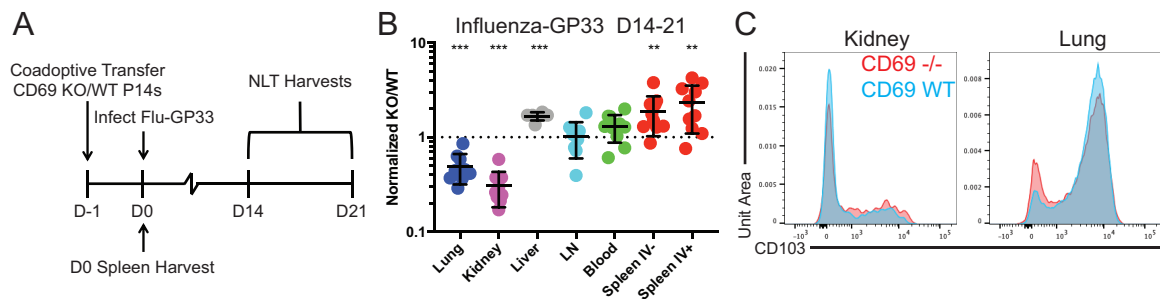


FIGURE 2.6 Lack of CD69 impairs T_{RM} formation in the lungs and kidney following influenza infection

Animals received a co-adoptive transfer of congenically distinct CD69^{-/-} and WT CD8⁺ P14 T cells, followed by infection with influenza-gp33 one day later (A). The ratio of co-transferred, IV- cells isolated from a variety of different tissues between 14 and 21 days after infection with influenza-gp33 (B). Surface expression of CD103 in the kidney and Lungs 14 days p.i. with influenza-gp33 (C). Error bars show mean \pm SD, one tailed t test against the known ratio of transferred cells on log₁₀ transformed data.

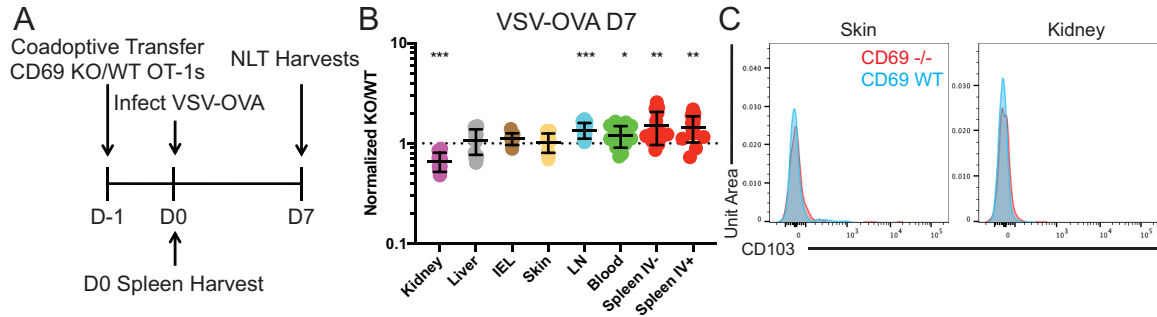


FIGURE 2.7 Absence of CD69 does not impair skin residency after VSV infection

Animals received a co-adoptive transfer of congenically distinct *Cd69*^{-/-} and WT CD8⁺ OT1 T cells, followed by infection with VSV-OVA one day later (A). The ratio of co-transferred, IV⁻ cells isolated from a variety of different tissues 7 days after VSV-OVA (B). Surface expression of CD103 in the skin and kidney 7 days p.i. with VSV-OVA (C). Error bars show mean \pm SD, one tailed t test against the known ratio of transferred cells on log₁₀ transformed data.

It was possible that the role for CD69 in producing T_{RM} within the kidney might relate to the P14 TCR specificity: it has been proposed that P14 T cells recognize a self-epitope expressed in the adrenal gland, which is proximate to the kidney (97, 98). Also, chronic infections with LCMV tend to persist in the kidney, potentially implying preferential tropism for this tissue (99). To address this, we extended our data to another TCR transgenic model and another pathogen, testing the response of WT and *Cd69*^{-/-} OT-I TCR transgenic CD8⁺ T cells, following infection with VSV-OVA (Fig. 2.7A). As for the studies with P14 T cells, CD69-deficiency resulted in reduced OT-I recruitment to the kidney, but there was no substantial affect in other NLTs tissues, including the skin (Fig. 2.7B, C).

Since unexpected viral tropism or immune clearance mechanisms might account for the repeated pattern of CD69-dependency in kidney T_{RM} generation, we substituted a peptide vaccination approach. P14 T cells were primed using “TriVax” immunization (comprising the LCMV gp33 peptide, poly-I:C and agonistic anti-CD40 antibody (100)) (Fig. 2.8A). Similar to the other immunization methods, *Cd69*^{-/-} P14 showed impaired accumulation in the kidney, but not the liver and spleen (Fig.2.8B, C).

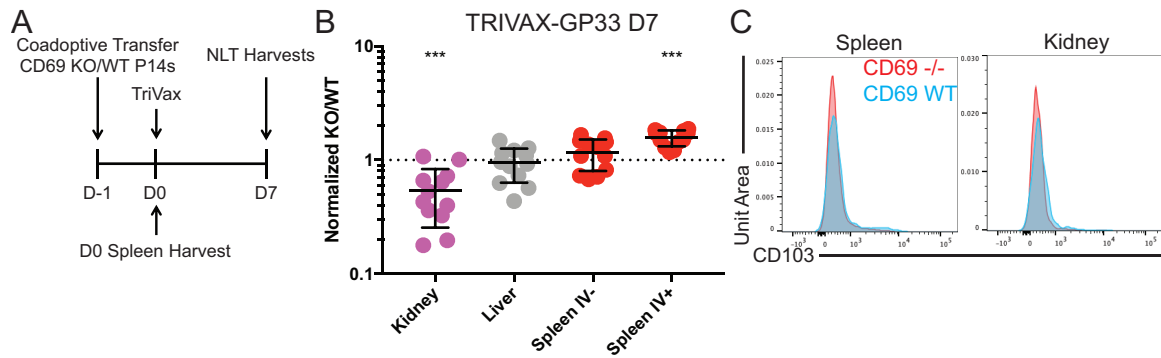


FIGURE 2.8 Pattern of tissue residency following peptide vaccine

Animals received a co-adoptive transfer of congenically distinct CD69^{-/-} and WT CD8⁺ P14 T cells, followed by administration of the TriVax peptide subunit vaccine one day later (A). The ratio of co-transferred, IV- cells isolated from a variety of different tissues 7 days after TriVax (B). Surface expression of CD103 in the spleen and kidney 7 days post TriVax (C). Error bars show mean \pm SD, one tailed t test against the known ratio of transferred cells on log₁₀ transformed data.

2.3 Discussion

The results presented here are the most comprehensive look at CD69's impact on the establishment and maintenance of T_{RM} to date. Using the coadoptive transfer model we investigated CD69's role in T_{RM} formation under a variety of conditions. The most notable finding was that in many situations the absence of CD69 did not impair the formation or maintenance of T_{RM} in a variety of NLT. In the small intestine intraepithelial (SI-IEL), lamina propria (SI-LP), and salivary gland CD69 simply was not required for tissue residency after an LCMV infection. These data represented a direct contradiction to the accepted theory of T_{RM} development. Quoting from Janeway's Immunobiology, "In these [epithelial] sites, T_{RM} induce CD69, which reduces S1PR expression, thereby promoting retention in tissues" (101). These data demonstrated that CD69 was entirely dispensable for tissue residency in sites where expression of KLF2 and downstream S1PR1 has been shown to be a severe detriment (69). Others in the field have acknowledged that CD69's role may be overstated due to the mild impact of CD69 deficiency in some models (80), but a situation where CD69 had no effect on residency had yet to be uncovered.

Some previous studies did not or were unable to make use of the ratio of WT and CD69-deficient transferred cells as a point of comparison, instead opting to use the ratio of cells in the spleen as a reference population (73, 88). We consistently observed an advantage for *Cd69*^{-/-} cells in the spleen, implying that previous work may have exaggerated the impact of CD69's absence in non-lymphoid sites.

The kidney represents a site where CD69's absence affected tissue residency regardless of the model employed. Our experiments with influenza show that local infection is not necessary for CD69 to affect tissue residency. Experiments with the TRIVAX peptide vaccine lead to a similar conclusion.

Because the TRIVAX vaccine is a peptide vaccine and therefore is unlikely to form antibody complexes the TRIVAX experiments also led us to conclude that an antigen depot established by antibody complexes cleared in the kidney (102) is not responsible for the residency defect universally seen in the kidney. Finally, the similar outcome in studies with OT-I T cells preclude the possibility that the P14 TCR transgenic model was responsible for the kidney's proclivity for CD69 expression. Results with the VSV-OVA model also highlighted the unpredictability of CD69's role in tissue residency. Previous work on CD69 focused heavily on the skin, with CD69 being important for skin tissue residency following a HSV infection or intradermal injection (67, 87). We repeated the latter result, but in the context of a VSV infection, we saw that the absence of CD69 did not impair tissue residency in the skin. This leads to an obvious question, what part of the context provided by an HSV infection is different from a VSV infection? The most obvious answer is the chronic nature of an HSV infection, and this is a good candidate for future studies.

Finally, we characterized CD69's impact on the phenotype of T_{RM} , both in the steady state and following in vivo reexposure to antigen. We observed no meaningful differences between CD69 deficient and sufficient cells in terms of their ability to express effector cytokines and chemokines implying that CD69 deficient cells are just as capable of exerting the sentinel (11) and effector (9) functions attributed to T_{RM} . Taken as a whole, these results strengthen the conclusion that CD69 promotes recruitment and retention of resident cells at sites of local infection, as well as in the kidney regardless of immunization strategy, but that CD69-deficiency has little consequence for the establishment or maintenance of T_{RM} at some other tissue sites, such as the SI-IEL, and that, in CD8+ T_{RM} , CD69-deficiency has little impact on processes other than localization.

2.4 Methods

2.4.1 Mice

C57BL/6 and B6.SJL mice were purchased from the National Cancer Institute. CD69 deficient mice were provided by Dr. Linda Cauley at the University of Connecticut (88) and crossed to P14 and OT1 TCR transgenic animals, as well as onto the C57BL/6 background. These mice were originally derived by Dr. Toshinori Nakayama at the University of Chiba.

2.4.2 Adoptive Transfers

A mixture of splenocytes containing 5×10^3 TCR transgenic cells at approximately a 1:1 ratio were co-adoptively transferred into recipients. C57BL/6 or B6.SJL recipients were used depending on the congenic markers of the transferred cells.

2.4.3 Pathogens and Infections

Where applicable, 2×10^5 lymphocytic choriomeningitis virus (LCMV) strain Armstrong was injected intraperitoneally (103). 1×10^6 Vesicular stomatitis virus expressing OVA (VSV-OVA), Indiana strain was injected intravascularly. 5×10^2 PR8 Influenza-gp33 (Flu-gp33) was applied intranasally following anesthesia with ketamine and xylazine. VSV-OVA (95) and Flu-gp33 (94) were provided by Dr. David Masopust at the University of Minnesota.

2.4.4 In Situ Peptide Stimulation of FRT T_{RM}

At least 30 days post coadoptive transfer followed by LCMV infection animals were challenged transcervically with gp33 peptide as in Beura et al (104). 12 hours later, lymphocytes were isolated from nonlymphoid tissues and analyzed by flow cytometry.

2.4.5 TriVax Immunization

A mixture containing 250µg of gp33 peptide, 100µg of agonist αCD40, and 50µg of Poly I:C was injected into the tail veins of recipient mice. Protocol modified from Cho et al (100).

2.4.6 *In Vitro* Activation followed by Intradermal Cotransfer

Splenocytes were isolated from CD69 sufficient and deficient P14 TCR transgenics and separately activated in vitro with plate-bound αCD3 and αCD28 as well as 10ng/ml IL-2 for 72 hours at 37° C. At 48 hours, media was changed, and cells were moved to new plates without αCD3 and αCD28. After the 72-hour incubation, an approximately 1:1 mixture of cultured cells containing 1x10⁶ total cells was made and was intradermally transferred into congenically distinct recipient animals. 9 days after the intradermal transfer, animals were euthanized, and lymphocytes were isolated from the skin and other tissues.

2.4.7 Isolation of Lymphocytes from Nonlymphoid Tissues

To differentiate cells in circulation from those in nonlymphoid tissues, mice underwent the IV labeling procedure as previously described (90), with a 5 minute gap between IV antibody injection and euthanasia. Lung, kidney, and salivary gland were digested with collagenase I and buffers as previously described (69), with the following alterations. Lung was incubated with collagenase I and digestion buffer for 60 minutes. Additionally, following enzymatic digestion, but prior to Percoll purification all of these tissues were ground using a gentleMACS tissue dissociator, using the program “Spleen1” twice. Cells were isolated from the liver using the gentleMACS tissue dissociator, followed by percoll purification, as in other tissues. Cells were isolated from the IEL, LP, spleen, and inguinal lymph nodes as previously described (69).

Lymphocytes were isolated from the skin and female reproductive tract as described by Beura et al (10).

2.4.8 Statistical Analysis

Data were analyzed using Graph Pad Prism software (8.1.2) Where indicated, one tailed t test compared against the known ratio of transferred cells using \log_{10} transformed data. Normalization of results in experiments involving an adoptive cotransfer of unmanipulated cells, were performed by dividing each individual data point by the known transfer ratio for that experimental repeat. Two tailed paired and unpaired t tests were also used where indicated. * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$.

2.5 Publication and Contributions

Chapter modified (with permission) from the following published article:

Walsh, D. A. *et al.* The Functional Requirement for CD69 in Establishment of Resident Memory CD8⁺ T Cells Varies with Tissue Location. *J. Immunol.* ji1900052 (2019). doi:10.4049/jimmunol.1900052

Author Contributions:

DAW, DM, and SCJ designed the experiments. DAW, HBdS, LKB, CP, and SEH performed experiments. DAW and SCJ wrote the manuscript with input from all authors.

Chapter 3

Developing a New Model of CD69-Mediated Tissue Residency

3.1 Introduction

Our results described in the previous Chapter suggested that CD69's impact on tissue residency was more limited than previously had been thought. CD69 sometimes seemed to play a role in tissue residency, albeit a limited one. We and others (80) observed that even when CD69 does impact tissue residency CD69 expression was not an absolute requirement, simply an advantage. This conclusion was based on the magnitude of the residency defect. Interestingly, the magnitude of the defect seems to vary with the site of residency, challenge, and source of inflammation. For instance, the defect is larger in the kidney than the lungs after an influenza infection. Notably, the magnitude of the defect was smaller in the kidney after a VSV infection compared to an LCMV infection. Previous studies reported more substantial defects in generation of *Cd69*^{-/-} T_{RM} (67, 87, 88). Notably, a wide range of experimental approaches have been employed in these previous studies, including substantial differences in the numbers of adoptively transferred CD8⁺ T cells used to evaluate the role for CD69 (67, 73, 87, 88). It is well established that altering the number of naïve precursor T cells can affect the behavior of those cells during subsequent immune challenge (105, 106). We therefore performed experiments to test the effects of a range of precursor frequencies on CD69 mediated tissue residency.

It was also unclear why and how CD69 was affecting tissue residency. CD69 is best known for preventing signaling through the G protein-coupled receptor S1PR1 (29). There is evidence that inhibiting S1PR1 reverses the effects of CD69 deficiency (87), thus it has been taken for granted that inhibition of S1PR1 is the sole mechanism by which CD69 acts to promote tissue residency. Recently, however, myosin 9 and 12 have been identified as ligands for CD69 (48). Myosin 9/12 form net-like structures in the blood vessel, and CD69-myosin interactions have been proposed as part of the leukocyte adhesion cascade that helps cells transition from the blood into inflamed nonlymphoid tissues (49). These results point to CD69 having two distinct roles in establishing

tissue resident cells. We wanted to investigate whether the association of CD69 with S1PR1 versus other ligands (e.g. myosin 9/12) were more important for establishing T_{RM} . Ultimately, the results of our studies indicated that interactions with S1PR1 were responsible CD69-mediated tissue residency.

Apart from being consistently important for kidney residency, there was no single thread that explained when and why CD69 could play a role in T cell tissue residency. The transcription factor KLF2 drives expression of S1PR1, and its downregulation is required for cells to become established as tissue resident (69). Downregulation of both KLF2 and S1PR1 is a key aspect of the transcriptional profile of T_{RM} (70, 85). Just as CD69 binds to and removes S1PR1 from the cell surface, high levels of S1PR1 will prevent CD69 from reaching the cell surface (69). We developed two models for CD69's role in establishing T_{RM} . In the first model, CD69 gets actively upregulated at some point during or soon after tissue entry. It prevents S1PR1 from reaching the cell surface, and thus actively promotes tissue residency. The second model frames CD69 as a passive marker of T_{RM} rather than an active player in establishing them. In this model, KLF2 and S1PR1 get downregulated quickly following tissue entry and because S1PR1 is no longer on the cell surface CD69 begins to appear there. However, our data described in Chapter 2 suggested that the role of CD69 seemed to be somewhere between these two extremes. Our previous results show that the magnitude of CD69-mediated residency was elastic, and that CD69 deficient cells were still capable of (if disadvantaged at) forming T_{RM} . One caveat to previous studies on the impact of CD69 deficiency (including the studies described in chapter 2) has been the use of intravenous (IV) labeling to identify T_{RM} as opposed to the more stringent technique of parabiosis. Parabiosis is considered the gold standard for identifying truly tissue resident cells, whereas IV labeling can overestimate the number of resident cells (107, 108). To address the validity of our models, we made use of parabiosis studies to examine the impact of CD69 on tissue residency more closely. We then made use of

CRISPR/Cas9 derived CD69 knockouts in KLF2 reporter mice to get a better understanding of KLF2 expression by CD69 deficient cells. These results helped us refine our model for CD69's role in establishing and maintaining T_{RM}.

3.2 Results

3.2.1 Naïve CD8⁺ T cell Precursor Frequency can Alter the Defect in Residency

The number of naïve antigen specific cells present prior to infection, or precursor frequency, was the first variable we tested. To test the impact of the naïve precursor frequency, we co-transferred *Cd69*^{-/-} and CD69 WT P14 CD8⁺ T cells of varying precursor number (between 5×10^2 and 5×10^5 P14 cells) into naïve recipients followed by LCMV infection. Seven days later, lymphocytes from a variety of different nonlymphoid tissues were isolated. Increasing the naïve

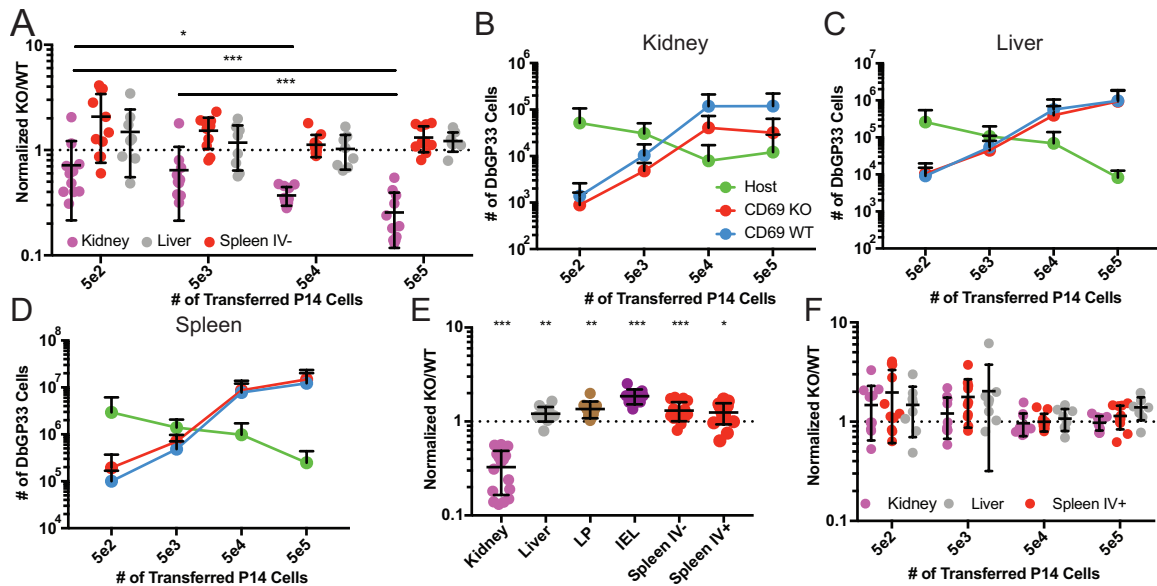


FIGURE 3.1 The frequency of antigen specific cells present prior to infection correlates with the magnitude of the defect in the kidney, but not elsewhere

A range of precursor cell numbers were co-adoptively transferred into recipient animals who were then infected with LCMV. The normalized ratio (*Cd69*^{-/-}/*Cd69* WT) of IV-transferred cells in each organ 7 days after infection with LCMV (A). The number of IV-cells 7 days p.i. in the kidney (B) and liver (C). The number of IV- and IV+ cells 7 days p.i. in the spleen (D). Also shown are endogenous D^bGP33-Tet⁺ cells. Normalized ratio of IV- *Cd69*^{-/-}/WT cells isolated from the indicated tissues 7 days p.i. with LCMV, following co-adoptive transfer of 5×10^5 total cells (E). Normalized ratio of IV+ over *Cd69*^{-/-} over WT cells isolated from the indicated tissues 7 days p.i. with LCMV, following co-adoptive transfer of between 5×10^2 and 5×10^5 cells as indicated on the x axis. Ratio of transferred cells indicated by the dotted line (F). ANOVA with multiple comparisons on log₁₀ transformed data for (A), one tailed t test against the known ratio of transferred cells on log₁₀ transformed data for (E).

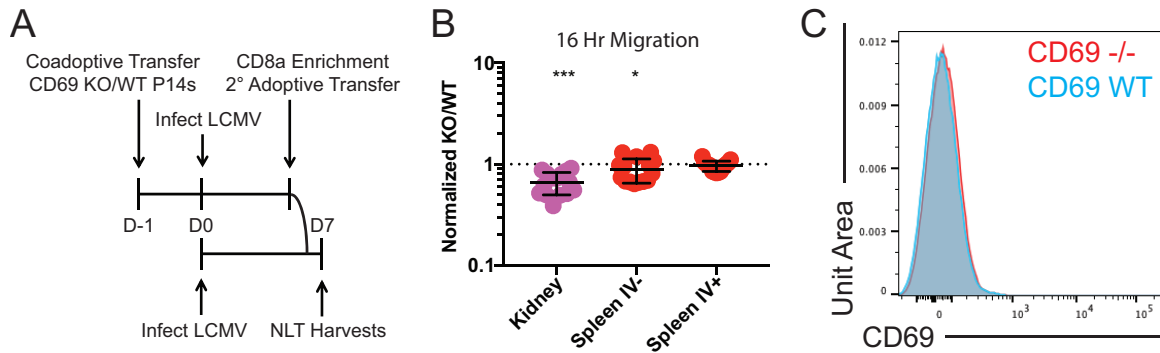


FIGURE 3.2 CD69 impairs migration into the kidney within a 16 hour window but the magnitude of its impact is reduced

Cd69^{-/-} and WT P14s were co-adoptively transferred as before, followed by LCMV infection. 6 days later, 5 million CD8⁺ T cells were purified from spleens of infected animals by magnetic enrichment and adoptively transferred into infection matched recipient animals (A). Graphed is the normalized ratio of IV- cells in the indicated tissues 16 hours after the secondary co-transfer (B). CD69 expression on cells directly prior to secondary co-transfer (C) One tailed t test against the known ratio of transferred cells on log₁₀ transformed data for (B).

precursor frequency significantly increased the magnitude of the residency defect observed for *Cd69*^{-/-} P14 CD8⁺ T cells in the kidney (Fig. 3.1A-D). Adoptive transfer doses of 5x10² and 5x10³ that lead to engraftment of P14 in the physiological range of endogenous gp33/D^b specific T cells (106), however, revealed less substantial effects of CD69-deficiency. Notably, increasing the precursor frequency did not alter the relative representation of *Cd69*^{-/-} T cells in other tissues or in the IV+ fraction (Fig 3.1E, F). These data suggest that use of non-physiologically high adoptive transfer studies may artificially exaggerate the significance of CD69 on establishing residency, at least in some tissues.

3.2.2 CD69 enhances early recruitment to the kidney

While we found that CD69 deficiency reduced the frequency of resident CD8⁺ T cells in the kidney at effector and memory phases of the immune response, it was not possible to determine whether the role of CD69 was primarily in initial recruitment to the site or the result of sustained retention. To explore this, we modified a previously described model of early T cell trafficking to the kidney (68). Mice received co-adoptive transfers of antigen specific WT

and *Cd69*^{-/-} P14 CD8⁺ T cells followed by LCMV infection. Six days post infection, we purified CD8⁺ T cells from the spleens of infected host mice and re-transferred them into infection matched hosts (Fig. 3.2A, B). Of note, neither population expressed CD69 on the cell surface at the time of transfer (Fig 3.2 C). In secondary hosts, *Cd69*^{-/-} cells were disadvantaged at entering and being retained in the kidney, though the magnitude of the residency defect was less than observed in figure 2.1 (Fig. 3.2B). This indicates that CD69 can act within a 16-hour window to promote tissue entry and/or short-term retention in the kidney. Hence, in keeping with previous models, these data suggest that contributions of CD69 to CD8⁺ T cell tissue residency are primarily reflected in initial recruitment.

3.2.3 Forced CD69 expression enhances residency in some tissues

Similar to previous reports (67, 73, 87, 88), our studies focused on defining the role played by CD69 in tissue residency through analysis of *Cd69*^{-/-} CD8⁺ T cells. However, an important corollary to this approach is to test whether ectopic CD69 expression might promote generation of tissue-resident cells. To address this, we performed retroviral transduction of in vitro activated, *Cd69*-sufficient P14 cells to force expression of CD69, prior to adoptive transfer and in vivo priming with LCMV (Fig. 3.3A). This approach does not lead to substantial CD69 overexpression (Fig 3.3B), but rather uncouples *Cd69* gene expression from normal transcriptional control. Compared to empty vector-transduced cells, P14 with forced expression of CD69 were enriched in the kidney (Fig. 3.4A-F). In other NLTs, there was often a slight trend towards enrichment of CD69 transduced cells, but it was minor, and not statistically significant. These data illustrate that for some NLT, establishment of CD8⁺ T cell residency does not detectably require CD69 yet enforced CD69 expression can improve generation of the resident population. Although significant, enhanced recruitment of *Cd69*-vector transduced cells in all tissues was relatively slight, in line with the reciprocal effects of CD69-deficiency. The retroviral transduction approach

allowed us to address the functional basis by which CD69 can promote residency in some tissue sites, through use of *Cd69* mutants. While CD69 is a C-type lectin and has been proposed to have several ligands, its best-defined interaction is with S1PR1, leading to mutual inhibition of cell surface expression. Indeed, earlier studies suggested that the role of CD69 in promoting CD8⁺ T cell tissue residency could be mimicked by pharmacological blockade of S1PR1 (87). To more specifically define the role of CD69-S1PR1 interactions in controlling CD8⁺ T cell tissue residency, we tested the effect of forced expression of a CD69 mutant, (6N6-Δ31, called CD69-Δ31 here), which does not interact with S1PR1 due to a domain swap of the CD69 transmembrane domain, but does not alter

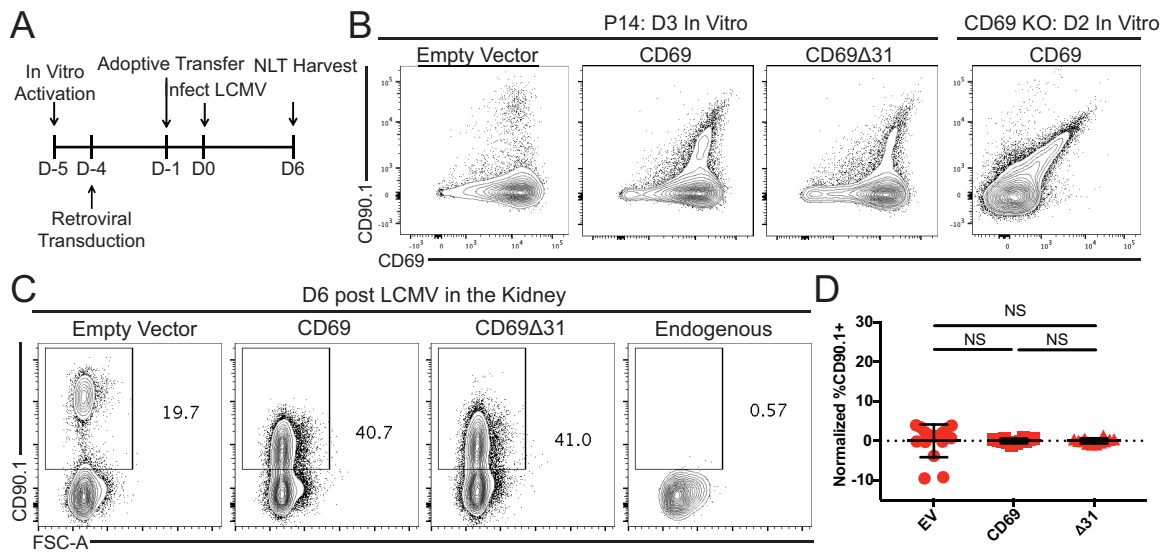


FIGURE 3.3 Retroviral transduction to understand the mechanism of CD69 mediated tissue residency

P14 CD8⁺ T cells were activated *in vitro* with αCD3 and αCD28 for 24 hours followed by transduction with retroviral vectors containing either empty vector, CD69, or CD69Δ31, which cannot interact with S1PR1. Cells then rested *in vitro* with IL-2 for 48 hours, followed by adoptive transfer into congenically distinct recipients (A). Expression of CD69 vs the transduction marker CD90.1 following transduction of CD69 WT or *Cd69*^{-/-} P14 CD8⁺ T cells. Expression of the transduction marker CD90.1 in empty vector, CD69, or CD69Δ31 transduced cells, in either *Cd69*^{-/-} or WT P14s where indicated *in vitro* (B). Expression of the transduction marker CD90.1 in the kidney 6 d.p. LCMV (C). The percentage of IV- transferred cells expressing the transduction marker CD90.1 on their cell surface in the spleen, normalized to the percent expressing it in the average of the spleens for each experimental repeat (D). ANOVA with multiple comparisons on log₁₀ transformed data for (B).

the C-type lectin, stalk or intracellular domains of CD69 (29). In contrast with cells transduced with vectors encoding WT CD69, P14 transduced with retroviruses encoding *Cd69-Δ31* failed to show enrichment in any NLTs (Fig. 3.4A-F). This is consistent with CD69-mediated S1PR1 regulation being the key mechanism that mediates enhanced generation of tissue resident cells, although alterations in interactions with other CD69 ligands by the $\Delta 31$ mutation cannot be excluded. Together these results indicate that, like CD69-deficiency, enforced expression of CD69 has only a limited effect on generation of tissue-resident CD8⁺ T cells.

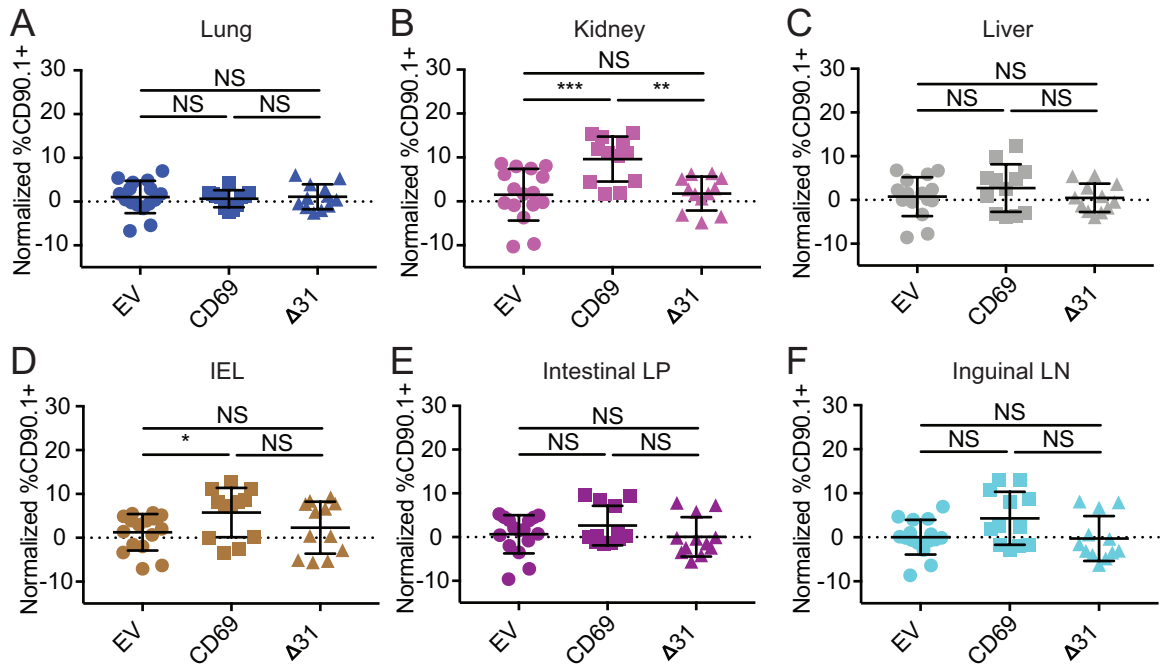


FIGURE 3.4 CD69 acts primarily via S1PR1 to promote tissue residency in the kidney

In vitro activated P14 cells were transduced retroviral vectors containing either empty vector, CD69, or CD69 $\Delta 31$, which cannot interact with S1PR1. Cells were transferred into hosts who were infected with LCMV the next day and nonlymphoid tissues were harvested 6 days post LCMV infection. Shown is the percentage of IV- transferred cells expressing the transduction marker CD90.1 on their cell surface, normalized to the percent expressing it in the average of the spleens for each experimental repeat in the lung (A), kidney (B), liver, (C) intestinal epithelium (D) intestinal lamina propria, (E) and inguinal lymph nodes (F). ANOVA with multiple comparisons.

3.2.4 CD69 deficiency impairs residency and appearance of KLF2^{low} CD8⁺ T_{RM} in the kidney

The defining feature of T_{RM} is their maintenance within tissues in the absence of recirculation. Parabiosis has been used to differentiate between resident memory and effector memory cells that transiently traffic through nonlymphoid tissues (69, 77, 104). To stringently assess how CD69 functionally impacts tissue residency, we used parabiosis to define the recirculation ability of CD69 sufficient and deficient cells in various tissues. Animals received a cotransfer of *Cd69*^{-/-} and WT P14s followed by LCMV infection. At memory timepoints, these parabiotic donor mice were surgically conjoined with infection matched parabiotic recipients that had not received P14 cells. At least 30 days later, the percent transferred cells in the blood (Fig 3.5A) and spleen (Fig 3.5B,C) was similar in donor and recipient parabionts. We used a formula adapted from Steinert et al. (77) to quantify the percent of *Cd69*^{-/-} or WT cells that were tissue

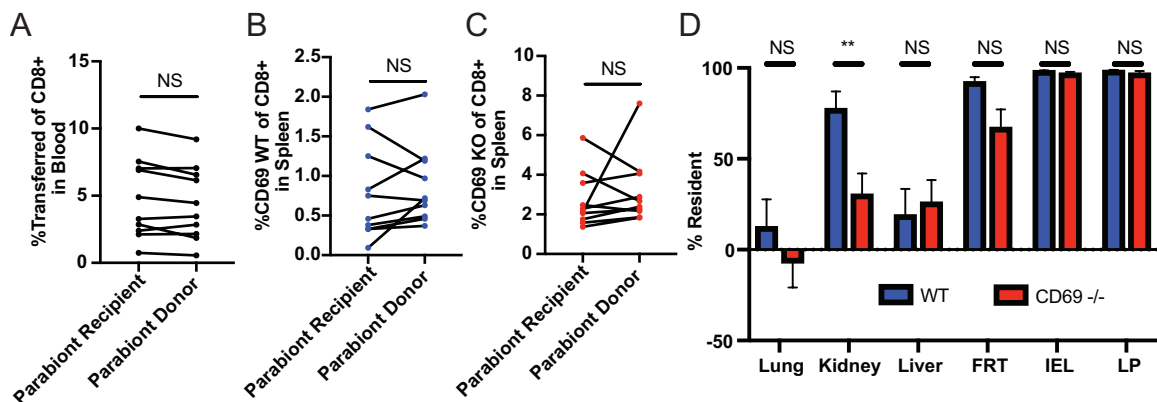


FIGURE 3.5 Parabiosis shows many *Cd69*^{-/-} cells in the kidney are not T_{RM}

Animals received a co-adoptive transfer of congenically distinct *Cd69*^{-/-} and WT P14 CD8⁺ T cells followed by LCMV infection. At a memory timepoint, these animals were surgically conjoined to infection matched animals and left to equilibrate for at least 30 days. At this point, cells were isolated from each of the indicated tissues with IV⁺ cells excluded from analysis for the nonlymphoid tissues. Shown is the percent of CD8⁺ T cells in the blood that were cotransferred in both the donor and recipient parabionts (A). The percent of CD8⁺ T cells in the spleens of parabiotic donor and recipient animals that were WT (B) or *Cd69*^{-/-} (C). The number of cells in the donor and recipient parabionts were used to calculate the percentage of cells that were tissue resident using methods from Steinert et al (D). Error bars show mean ± SEM for (D). Two tailed t test for (A-C) ANOVA with multiple comparisons for (D).

resident (Fig 3.5D). As expected, the frequency of resident cells detected by flow cytometry varied considerably with tissues, ranging from high (in the small intestine) to very low (in the lung). The impact of CD69-deficiency on residency also differed with tissue site, but in a distinct pattern. Thus, in the SI-IEL and SI-LP, CD69 sufficient and deficient cells exhibited a similar very high degree of residency. Likewise, although few liver cells were resident, this was no different for *Cd69*^{-/-} or WT cells. In the kidney, however, there was a clear and significant residency defect for *Cd69*^{-/-} cells. Furthermore, *CD69*^{-/-} P14 showed a trend of reduced residency in the FRT, although this was not statistically significant. Hence, the impact of CD69 deficiency on tissue residency varies considerably across non-lymphoid tissues.

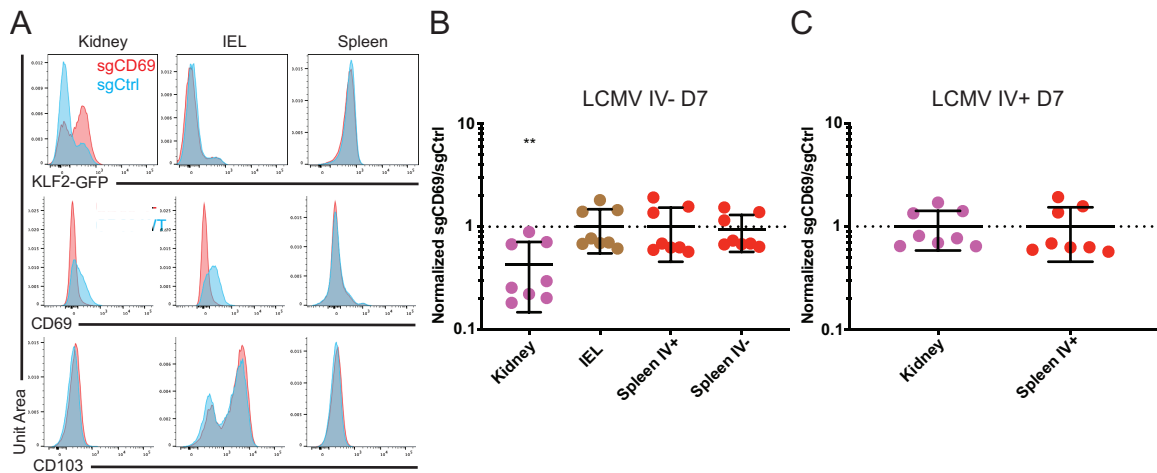


FIGURE 3.6 Using CRISPR/Cas9 to knockout CD69 gives a similar phenotype to germline knockouts

CRISPR/Cas9 was used to knockout CD69 in KLF2-GFP expressing P14 T cells. Animals received a cotransfer of these cells alongside the congenically distinct KLF2-GFP P14s that used a scramble sgRNA as a control, followed by LCMV infection. Expression of KLF2-GFP, CD69, and CD103 on cells that received CD69 sgRNA and cells that received scramble sgRNA 7 d.p.i. in the indicated tissues (A). The ratio of recovered CD69 sgRNA/scramble sgRNA receiving cells in the indicated organs 7 d.p.i. in the IV- (B) and IV+ (C) one tailed t test against the known ratio of transferred cells on log10 transformed data for (B and C).

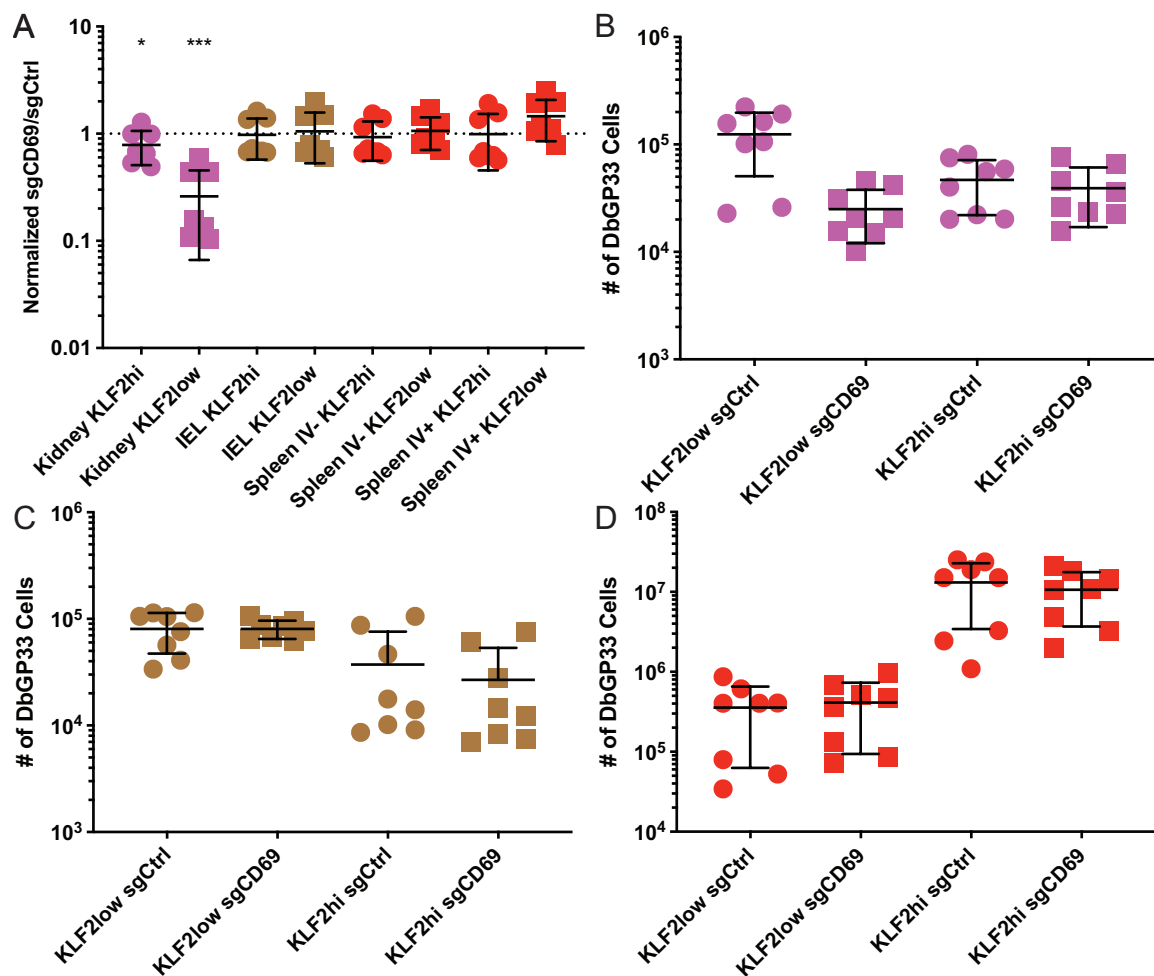


FIGURE 3.7 CD69-deficiency impairs tissue-residency and appearance of KLF2^{low} CD8⁺ T cells in the kidney

The ratio of recovered CD69 sgRNA/scramble sgRNA receiving cells in KLF2-GFP hi and low cells in the IV- fraction of the indicated organs 7 d.p. LCMV infection is shown in (A). The number of cells that received either CD69 or scramble sgRNA, separated by KLF2 expression 7 d.p.i. recovered from the kidney (B), intestinal-IEL (C), and spleen (D). One tailed t test against the known ratio of transferred cells on log₁₀ transformed data for (A).

Reduced KLF2 expression is a functionally relevant feature of tissue resident memory CD8⁺ T cells (69, 70, 85), prompting the question of whether the failure of kidney *CD69*^{-/-} P14 cells to establish residency would correlate with altered KLF2 expression. To test this, we used the CRISPR/Cas9 system to generate CD69 deficient, KLF2-GFP expressing P14 cells to explore the impact of CD69 deficiency on KLF2 expression and to further characterize *Cd69*^{-/-} cells

present in the kidney. Activated KLF2-GFP expressing P14s were treated with Cas9 and either CD69 sgRNA or scramble controls (109), cotransferred into recipient mice and infected with LCMV infection the same day. 7 days post infection, lymphocytes were isolated from nonlymphoid tissues. The absence of CD69 was confirmed on the surface of cells in nonlymphoid tissues (Fig 3.6A). Independent of KLF2-GFP expression the ratio sgCD69/sGCtrl closely mirrored the phenotype observed using germline knockouts (Fig 3.6B,C). Interestingly, CD69 deficient cells were no longer present at an elevated frequency in the spleen. In vitro activated germline knockouts presented with the same phenotype upon cotransfer (data not shown). In the spleen and IEL there was no difference in KLF2-GFP expression when comparing CD69 sufficient and deficient cells. In the kidney the KLF2^{low} population was severely ablated, while the KLF2^{hi} cells were intact (Fig 3.6A). This was apparent both by the ratio of cells (Fig 3.7A) and by cell numbers (Fig 3.7B-D). These data indicated that the residency defect in the kidney was only among KLF2^{low} cells (potentially slated to develop into tissue resident memory) and not in the KLF2^{hi} compartment. These data also reinforce the notion that reduced KLF2 expression is a defining feature of the tissue resident population.

3.3 Discussion

Taken as a whole these experiments resolve several important questions in the field and solidify a refined model of CD69-mediated tissue residency for CD8⁺ T_{RM}. The precursor frequency experiments identify an uncontrolled variable in the prior work of several groups which may explain the variations in the data from different laboratories studying CD69-mediated T_{RM}. The results of the short term migration re-transfer assays suggest that CD69 functions in the early stages of residency, and can act in a 16 hour window. Previous work concluded that CD69's impact on tissue residency is limited to establishment and maintenance during the effector phase of the immune response (87) but our re-transfer experiments further refined the window in which CD69 was capable of acting. Our work is consistent with CD69 promoting residency through S1PR1 inhibition (29), and not to binding to myosin 9/12 (48), but not dispositive. The fact that the magnitude of the residency defect is reduced in this short window of time is consistent with S1PR1 inhibition being the more important function because S1PR1 would be expected to promote tissue residency over a slightly longer window of time. This result strengthens a similar interpretation of our retroviral overexpression data, which show that S1PR1 inhibition is solely responsible for promoting tissue residency.

Our results indicate that, contrary to current dogma, CD69 expression plays no detectable role in establishment or maintenance of CD8⁺ T_{RM} in some NLT, such as the small intestine intraepithelial (SI-IEL) and lamina propria (SI-LP) populations while a substantial requirement for CD69 was observed for CD8⁺ T_{RM} generation in the kidney. CD69 expression moderately favored CD8⁺ T_{RM} establishment in the lung following influenza infection and the female reproductive tract following LCMV infection. This conclusion was suggested by the results of our cotransfer experiments, but the results of the parabiosis and CRISPR/Cas9 experiments solidify this conclusion. In complementary studies,

forced expression of CD69 enhanced generation of T_{RM} in the kidney but had minimal impact on induction of T_{RM} in the other NLT tested. Our studies reveal that, despite the near ubiquity of cell-surface CD69 as a feature of CD8⁺ T_{RM}, its functional relevance for establishing or maintaining tissue residency is highly variable across distinct non-lymphoid sites. This suggests caution in interpreting the presence or absence of CD69 expression as an indication of CD8⁺ T cell tissue-residency.

The results of the parabiosis and CRISPR/Cas9 experiments were particularly striking and led us to reevaluate our model of CD69's effect on tissue residency. Based on the relatively small size of the residency defect observed in our cotransfer experiments and the result of our precursor frequency experiments we assumed that CD69's impact on residency was graded. We imagined a model where the impact of CD69 fell somewhere between an absolute requirement for T_{RM} and not being important at all, and that this impact depended on context, like the infectious agent, tissue, or precursor frequency. Instead, the results of our parabiosis experiments indicated that measuring the ratio of cotransferred CD69-WT and *Cd69*^{-/-} cells substantially underestimated the true magnitude of CD69's impact on tissue residency – but only in cases where it was already clear that there was an impact. Specifically, a substantial number of CD69-deficient cells were present in the kidney at late memory timepoints but these were not functionally tissue resident, making it likely that these are circulating effector memory cells. We previously dismissed this possibility because the magnitude of the residency defect does not change over time, and we would have expected the effector memory population to change over time during contraction. The contradiction between these two threads of evidence does seem to indicate that this initial assumption was unfounded, and that contraction is similar in the T_{RM} and T_{EM} compartments. Further studies will be required to make this conclusion. Because loss of KLF2 is associated with tissue residency transcriptionally (69, 70, 85) and because it indirectly affects CD69 via S1PR1, we hypothesized that

different contexts that a cell auditioning to develop into T_{RM} can find itself in. This research implies that in tissues like the kidney, KLF2 and downstream S1PR1 are expressed by many cells fated to become T_{RM}. These cells require CD69 to transition into a fully resident population. It is likely that, in its absence, they are not maintained in the tissue long enough to receive residency cues from signals such as TGF- β (which can drive down expression of KLF2) (69). RNA-seq results from other groups show that after this transition period is over, KLF2 and S1PR1 are downregulated (70, 85). This implies that after a cell becomes established in the nonlymphoid tissue, it ceases to rely on CD69. In tissues like the intestine, by contrast, our work indicates that KLF2 and S1PR1 are not expressed after cells have entered the tissue, or are very quickly repressed. Because of this, tissue residency for CD8⁺ T cells in this tissue appears to be completely independent of CD69. These results suggest that it may be possible to predict the impact of CD69-deficiency by measuring KLF2 expression by cells auditioning for residency under different contexts. Understanding the factors that influence KLF2 expression in nonlymphoid tissues will be a focus of future studies.

3.4 Methods

3.4.1 Mice

KLF2-GFP expressing mice (110) were crossed to P14 TCR transgenic animals. Animals were maintained under specific-pathogen-free conditions at the University of Minnesota. All experimental procedures were approved by the institutional animal care and use committee at the University of Minnesota.

3.4.2 Adoptive Transfers

A mixture of splenocytes containing between 5×10^2 and 5×10^5 TCR transgenic cells at approximately a 1:1 ratio were co-adoptively transferred into recipients. When the precursor frequency is not specified 5×10^3 TCR transgenic cells were used. C57BL/6 or B6.SJL recipients were used depending on the congenic markers of the transferred cells.

3.4.3 In Vivo 16 Hour Migration Assay

A mixture of splenocytes containing 5×10^3 CD69 sufficient and deficient P14 cells at approximately a 1:1 ratio was co-adoptively transferred into congenically distinct recipients. The next day, recipients were infected with LCMV Armstrong. 6 days post infection, CD8⁺ T cells were purified via MACS negative selection from the spleens of recipients, and 5×10^6 CD8⁺ T cells were adoptively transferred into infection matched recipient animals. 16 hours after the adoptive transfer, the secondary recipients were euthanized, and lymphocytes were isolated from the kidney and other tissues. Protocol modified from Ma et al (68).

3.4.4 Retroviral Transduction of P14 Cells

CD8 T cells were purified by magnetic separation and subsequently activated in vitro with plate-bound α CD3 and α CD28 for 24 hours at 37° C. Cell cultures were then spininfected for 2 hours at 37° C with viral supernatant containing polybrene, β ME, and 10ng/ml mll-2. After spininfection, cells rested for

48 hours. Cells were then adoptively transferred into congenically distinct recipient animals, who were infected with LCMV Armstrong one day later. Adoptive transfers contained 1 million total cells, between 3% and 36% of which were transduced with empty vector, CD69, or CD69 Δ 31 (29) as determined by the transduction marker CD90.1. Plasmids encoding these constructs were kindly provided by Dr. Jason Cyster (UCSF). Transduced cells were adoptively transferred either alone, or in competition with congenically distinct empty vector transduced cells. Protocol modified from Skon et al (69).

3.4.5 Parabiosis

C57Bl/6J mice were co-transferred with CD69 sufficient and deficient P14 cells at approximately a 1:1 ratio one day prior to being infected with LCMV. A separate group of mice received no transgenic T cells but were infected with LCMV to create infection-matched mice. At least 30 days post infection the P14 transgenic mice were surgically joined along the flank to the infection-matched non-P14 bearing mice. Parabiosis surgery was performed as described in Beura et al (104). Equilibration of circulating T cell population was confirmed in the peripheral blood of conjoined pairs 4 week post-surgery. Parabiotic pairs were euthanized and lymphocytes were harvested from indicated tissues 29 or 38 days post-surgery. The formula from Steinert et al. (77) was used to calculate percent residency, as reported in Fig. 3.5.

3.4.6 CD69 Knockout via CRISPR/Cas9

KLF2-GFP P14 CD8⁺ T cells were isolated by magnetic separation and activated with plate bound α CD3 and α CD28 (both 5ug/ml) and 100 U/ml rhIL-2 for 48 hours at 37° C. The next day a 3:1 mixture of sgRNA with Cas9 was made and incubated at 20° C for 10 minutes prior to nucleofection using a Lonza 4D-Nucleofector X Unit (program CM137) per Seki and Rutz (109). Congenically distinct cells received sgRNA targeting either *CD69*

(CAUUUUGAGAAGCAUCAUGA and CAUCACGUCCUUAUAAUAG) or a scramble control (GCACUACCAGAGCUAACUCA). Cells were allowed to recover for 2 hours at 37°C before being transferred to a 24-well plate with 100 U/ml rhIL-2 for 2 days. Approximately 40 hours post transfection 5×10^4 of each cell type (CD69/scramble sgRNA) were coadoptively transferred into congenically distinct B6 recipient animals, followed by LCMV infection one hour later. Aliquots of cells were sequenced 7 days post electroporation and “ICE” scores calculated (ice.synthego.com) to determine targeting efficiency. ICE scores ranged from 83 to 86. Effective knockout efficiency was determined via protein expression in vivo.

3.4.7 Statistical Analysis

Where applicable, results were analyzed as described in chapter 2. Normalization of results in experiments involving transduction with retroviral vectors was performed by calculating the displacement of the percent of transduced cells in each individual sample from the average percent of cells transduced in the spleen. Splenic averages were calculated separately for each experimental repeat. The gate defining transduced cells (CD90.1+) was set on congenically distinct populations of either endogenous host cells or cotransferred empty vector transduced cells where appropriate. Because the gate defining transduced cells varied from tissue to tissue, ANOVA with multiple comparisons was only used to compare within tissues. * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$. Error bars show mean \pm SD unless otherwise indicated.

3.5 Publication and Contributions

Chapter modified (with permission) from the following published article:

Walsh, D. A. *et al.* The Functional Requirement for CD69 in Establishment of Resident Memory CD8⁺ T Cells Varies with Tissue Location. *J. Immunol.* ji1900052 (2019). doi:10.4049/jimmunol.1900052

Author Contributions:

DAW, DM, and SCJ designed the experiments. DAW, HBdS, LKB, CP, and SEH performed experiments. DAW and SCJ wrote the manuscript with input from all authors.

Chapter 4

Conclusion

The proposed functional role of CD69 expression as being critical for establishment of memory T cell tissue residency has been widely embraced, reaching the level of acceptance in immunology textbooks (101). Our data, however, argue against such a universal role for CD69. Rather, the functional significance of CD69 expression varies considerably with tissue site and immunization model. We also found that non-physiological assays, such as direct injection of activated CD8⁺ T cells into NLT and adoptive transfer experiments involving high numbers of naïve precursors may exaggerate the impact of CD69 in at least some tissues sites. Hence, we conclude that a non-redundant functional role of CD69 in dictating CD8⁺ T cell tissue residency is modest or absent for several non-lymphoid tissues.

We confirmed a role for CD69 in generating resident cells in the lung following influenza infection, consistent with other studies involving local infections (67, 73, 87, 88). These results may suggest that residual antigen and/or inflammation localized at the site of infection promotes or sustains a CD69-dependent recruitment or retention process. Even so, the impact of CD69-deficiency in the influenza-infected lung was relatively modest, as has been reported previously (73). It is possible that a functional role for CD69 may be stronger in situations of chronic infection, and it is notable that the most dramatic requirement for CD69 in driving CD8⁺ T cell residency comes from studies of the response to persistent HSV infection in the skin (67), but further studies will be required to compare the functional role for CD69 in situations of sustained versus transient antigen exposure.

On the other hand, a substantial impairment in generation of *Cd69*^{-/-} T_{RM} was observed in the kidney regardless of the immunization strategy (including use of the TriVax sub-unit vaccine), suggesting that local infection is not the only circumstance where CD69 expression guides T cell tissue residency. Indeed, CD69-deficiency led to a reduction in the development of a tissue-resident population in the kidney (as defined by parabiosis), while *CD69*^{-/-} CD8⁺ T cell

residency was unimpeded in the SI-IEL and SI-LP pools. This corresponded with defective recruitment of KLF2^{low} CD8⁺ T cells to the kidney following CD69 ablation. In previous studies, we showed that reduced expression of KLF2 and its transcriptional target S1PR1 was necessary for generation of CD8⁺ T_{RM} in many non-lymphoid sites (69). Hence there are two ways in which S1PR1 protein expression might be limited on T_{RM} – one involving loss of *S1pr1* transcription (typically following loss of KLF2 expression), and the other associated with upregulation of CD69 which can impede cell-surface expression of S1PR1 protein. Although one could imagine that these pathways could operate independently, it is noteworthy that in the case of the kidney, where we find a clear-cut requirement for CD69 in establishing tissue-residency, CD69-deficiency is not compensated by an increased frequency of KLF2^{low} cells. To the contrary, KLF2^{low} cells were virtually absent in the rare *Cd69*^{-/-} cells from the kidney parenchyma. This might imply that, for the kidney, CD69 induction is a necessary pre-requisite for down-regulation of KLF2, as was previously proposed for skin T_{RM} (87). For other tissues, such as the SI-IEL, however, it appears that KLF2-downregulation is entirely independent of the ability of the T_{RM} precursors to express CD69.

It is unclear why formation of the kidney T_{RM} pool would so consistently and substantially depend on CD69 expression. Interestingly, S1PR1 and its ligand S1P are important for renal function, including sodium secretion (natriuresis) (111–113). Hence, while S1P is degraded in the parenchyma of most tissues (28, 82), there may be areas of the kidney that maintain elevated levels of S1P. In such a scenario, blocking cell-surface S1PR1 through CD69 upregulation on circulating T cells, might provide a key advantage to avoid responding to S1P in the kidney. While it is of course possible that the contribution of CD69 to residency in the kidney is independent of its interactions with S1PR1, the fact that the CD69-Δ31 mutant, which retains the entire extracellular domain of WT CD69, fails to enhance CD8⁺ T cell residency in the

kidney makes the role for alternative ligands less likely. TCR (32) signal and cytokines such as TGF- β (69) are all capable of downregulating expression of KLF2. Other groups have established that TCR signals are important for establishing T_{RM}, especially in sites like the lungs (96) and skin (64). Likewise, the presence of TGF- β receptor on auditioning CD8+ T cells is important for establishing T_{RM} (68). These lines of evidence raise the possibility that tissue signals (including but not limited to TGF- β and TCR signals) repress KLF2 over time as a CD8+ T cell auditions for tissue residency. The presence and relative amounts of each of the different factors could be what dictates a requirement for CD69. In nonlymphoid tissues with fewer KLF2 repressing signals, CD69 would play a larger role. If this is the case, it may be possible to predict the impact of CD69 by looking at KLF2 expression on cells auditioning for residency. We would predict KLF2 to be expressed at higher levels and/or longer by T cells in the kidney, and to be suppressed quickly after tissue entry in the small intestine. This tissue specific context could be informed by many different variables. One possibility is the tissue parenchyma serving as a source of T_{RM} promoting cytokines (114). Likewise, the presence of E- and P-selectins in the blood vessels varies with the NLT (45, 46). The context provided by the innate immune system remains the most likely candidate, however. There is evidence that interactions with innate immune cells serves to maintain T_{RM} (65), and that the absence of such cells prevents T_{RM} from ever becoming established in the first place (115). It is possible that interactions with innate immune cells shortly after entering a nonlymphoid tissue license a cell to become resident. Antigen presentation, bystander cytokines, or some mixture of the two could be responsible for this. Perhaps the absence of CD69 prevents nascent T_{RM} from forming productive interactions with antigen presenting cells immediately after entering some nonlymphoid tissues. This hypothesis would predict that innate immune subsets would differ between NLTs. Given their role as professional antigen presenting cells macrophages and dendritic cells are the most likely

candidates for a difference between, for instance, the kidney and small intestine. Alternatively, the localization of innate cells may be distinct in different NLT. Regardless of the mechanism, the contribution of innate immune subsets to the establishment of T_{RM} remains an understudied area, and should be a topic of future research.

Interactions with innate immune system and anatomical quirks of the kidney are attractive explanations for CD69's seemingly random proclivity for particular residency contexts. One alternative is that CD69's role in tissue residency is an evolutionary holdover. CD8⁺ T cells are not the only immune subset that are classified as tissue resident. ILCs, macrophages, and $\gamma\delta$ T cells also have tissue resident subsets (116). In contrast to adaptive immune T_{RM} , however, tissue residency in these cells is regulated developmentally. For instance, the dendritic epidermal population of $\gamma\delta$ T cells is seeded into the skin of mice at embryonic days 14-16 and these cells are thought to perform an innate like immune function, recognizing keratinocyte stress antigens (117). A recent report concluded that CD69 was important for the retention of developmentally seeded $\gamma\delta$ T cells, and the maintenance of keratinocyte health (118). Likewise, CD69 expression by CD4⁺ T cells mediates improved antibody affinity by improving T follicular helper cell function (119) and biases cells towards a T regulatory cell fate by simultaneously inhibiting S1PR1 and promoting STAT5 signaling (120). Apart from these specific examples, CD69 is expressed by a wide variety of immune subsets, and is used as an early marker of activation on natural killer, B cells, monocytes, neutrophils and eosinophils (121). Interestingly, CD69 is differentially regulated by T and B cells, and more strikingly, by mature T cells and thymocytes with different promotor elements regulating its expression in different cell types (122). CD69's widespread pattern of expression is consistent with it appearing early during the evolution of the immune system. CD69 is hypothesized to have diverged from a family of proteins which includes many

natural killer cell markers (123). It may be that CD69 expression in some subsets is simply vestigial, though this is clearly not the case for all CD8⁺ T_{RM}.

Our studies demonstrated that *Cd69*^{-/-} CD8⁺ T cells are present at elevated frequencies in the spleen (and, in some situations, the blood and lymph nodes). The basis for this finding is unclear but may indicate that loss of basal CD69 expression promotes S1PR1-mediated recruitment or retention in the spleen. There is precedent for altered lymphoid tissue localization of *Cd69*^{-/-} cells. Others have reported that CD69-deficient CD4⁺ T cells cannot enter the bone marrow, and this entry defect prevents them from inducing high affinity antibodies produced by bone marrow resident plasma cells (119). It may be that the localization of CD69-deficient CD8⁺ T cells is altered in the spleen or lymphoid organs in an analogous fashion, but the techniques we used were not capable of resolving this question. Regardless of the mechanism, this result may further exaggerate reports of impaired NLT residency by *Cd69*^{-/-} CD8⁺ T cells, since *Cd69*^{-/-}/WT ratios in the spleen have often been used as a reference tissue in these studies.

Consistent with other proposed models (67, 87), our data suggest that any discernable role for CD69 in regulating CD8⁺ T cell residency in NLT residency is manifest early in the immune response, at least in the models of acute infection/immunization studied here. We did not find that effects of CD69-deficiency became more or less marked when studying tissue-associated cells at the effector phase versus T_{RM} at long-term memory time points. Likewise, we were able to show that CD69-dependent recruitment to the kidney could be measured in short-term (16h) homing assays. Together, these data suggest that a functional role of CD69, following acute immune responses, is likely to mediate initial recruitment and seeding of the NLT sites (87). Resident cells unable to make CD69 did not express higher levels of CD103 to compensate for CD69's absence. This reinforces the idea that CD69 and CD103 function at distinct timepoints. In seeming contrast with this, sustained cell-surface CD69 expression

is one of the most consistent features of long-term T_{RM} . Loss of S1PR1 expression (frequently associated with loss of KLF2 expression (69, 103)) may lead to cell-surface CD69 derived from basal expression in memory $CD8^+$ T cells. Still, one might expect that some $CD8^+$ T_{RM} may extinguish *Cd69* gene expression during their maintenance in NLT, if it does not play a functional role. In fact, there is existing evidence for this idea, since analysis of tissue-resident $CD8^+$ T cells by quantitative immunofluorescence and parabiosis indicates that a significant fraction of T_{RM} in various sites (pancreas, salivary gland, but also kidney) lack expression of CD69 (77). In addition, a meaningful fraction of CD69 expressing cells in lymphoid tissues recirculate (10). These studies could not discern whether CD69 expression was critical for initial seeding to those NLT but in conjunction with our findings, it is likely that a significant fraction of T cells that do not express CD69 or rely on CD69 for their establishment are indeed resident in NLT. Likewise, our studies using forced CD69 expression, following retroviral transduction, suggested relatively mild impact on recruitment of cells to NLT. Together, such findings suggest considerable caution is warranted in interpreting the significance of CD69 expression as an indication of tissue-residency by $CD8^+$ T cells in both lymphoid and non-lymphoid tissues. Although there is a growing understanding of the unique gene expression characteristics of T_{RM} versus recirculating cells, most of these studies used CD69 expression as an essential marker for identifying “resident” cells, and hence reinforce reliance on this molecule for their analysis. At least in mice, it would be better not to infer residency solely from CD69 surface expression.

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